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(57) Abstract <p>The invention provides a novel calcium-independent cytosolic phospholipase A₂/B enzyme, polynucleotides encoding such enzyme and methods for screening unknown compounds for anti-inflammatory activity mediated by the arachidonic acid cascade.</p>		

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CALCIUM INDEPENDENT CYTOSOLIC PHOSPHOLIPASE A₂/B ENZYMES

5 This application is a continuation-in-part of application Ser. No. 08/281,193, filed July 27, 1994.

The present invention relates to a purified calcium independent cytosolic phospholipase A₂/B enzymes which are useful for assaying chemical agents for
10 anti-inflammatory activity.

BACKGROUND OF THE INVENTION

The phospholipase A₂ enzymes comprise a widely distributed family of enzymes which catalyze the hydrolysis of the acyl ester bond of
15 glycerophospholipids at the sn-2 position. One kind of phospholipase A₂ enzymes, secreted phospholipase A₂ or sPLA₂, are involved in a number of biological functions, including phospholipid digestion, the toxic activities of numerous venoms, and potential antibacterial activities. A second kind of phospholipase A₂ enzymes, the intracellular phospholipase A₂ enzymes, also known as cytosolic
20 phospholipase A₂ or cPLA₂, are active in membrane phospholipid turnover and in regulation of intracellular signalling mediated by the multiple components of the well-known arachidonic acid cascade. One or more cPLA₂ enzymes are believed to be responsible for the rate limiting step in the arachidonic acid cascade, namely, release of arachidonic acid from membrane glycerophospholipids. The action of
25 cPLA₂ also results in biosynthesis of platelet activating factor (PAF).

The phospholipase B enzymes are a family of enzymes which catalyze the hydrolysis of the acyl ester bond of glycerophospholipids at the sn-1 and sn-2 positions. The mechanism of hydrolysis is unclear but may consist of initial hydrolysis of the sn-2 fatty acid followed by rapid cleavage of the sn-1 substituent,
30 i.e., functionally equivalent to the combination of phospholipase A₂ and lysophospholipase (Saito et al., *Methods of Enzymol.*, 1991, 197, 446; Gassama-Diagne et al., *J. Biol. Chem.*, 1989, 264, 9470). Whether these two events occur at the same or two distinct active sites has not been resolved. It is also unknown

if these enzymes have a preference for the removal of unsaturated fatty acids, in particular arachidonic acid, at the sn-2 position and accordingly contribute to the arachidonic acid cascade.

Upon release from the membrane, arachidonic acid may be metabolized via the cyclooxygenase pathway to produce the various prostaglandins and thromboxanes, or via the lipoxygenase pathway to produce the various leukotrienes and related compounds. The prostaglandins, leukotrienes and platelet activating factor are well known mediators of various inflammatory states, and numerous anti-inflammatory drugs have been developed which function by inhibiting one or more steps in the arachidonic acid cascade. Use of the present anti-inflammatory drugs which act through inhibition of arachidonic acid cascade steps has been limited by the existence of side effects which may be harmful to various individuals.

A very large industrial effort has been made to identify additional anti-inflammatory drugs which inhibit the arachidonic acid cascade. In general, this industrial effort has employed the secreted phospholipase A₂ enzymes in inhibitor screening assays, for example, as disclosed in U.S. 4,917,826. However, because the secreted phospholipase A₂ enzymes are extracellular proteins (i.e., not cytosolic) and are not specific for hydrolysis of arachidonic acid, they are presently not believed to participate directly in the arachidonic acid cascade. While some inhibitors of the small secreted phospholipase A₂ enzymes have anti-inflammatory action, such as indomethacin, bromphenacyl bromide, mepacrine, and certain butyrophenones as disclosed in U.S. 4,239,780, it is presently believed that inhibitor screening assays should employ cytosolic phospholipase A₂ enzymes which directly participate in the arachidonic acid cascade.

An improvement in the search for anti-inflammatory drugs which inhibit the arachidonic acid cascade was developed in commonly assigned U.S. Patent No. 5,322,776, incorporated herein by reference. In that application, a cytosolic form of phospholipase A₂ was identified, isolated, and cloned. Use of the cytosolic form of phospholipase A₂ to screen for anti-inflammatory drugs provides a significant improvement in identifying inhibitors of the arachidonic acid cascade. The cytosolic phospholipase A₂ disclosed in U.S. Patent No. 5,322,776 is a 110

kD protein which depends on the presence of elevated levels of calcium inside the cell for its activity. The cPLA₂ of U.S. Patent No. 5,322,776 plays a pivotal role in the production of leukotrienes and prostaglandins initiated by the action of pro-inflammatory cytokines and calcium mobilizing agents. The cPLA₂ of U.S. Patent
5 No. 5,322,776 is activated by phosphorylation on serine residues and increasing levels of intracellular calcium, resulting in translocation of the enzyme from the cytosol to the membrane where arachidonic acid is selectively hydrolyzed from membrane phospholipids.

In addition to the cPLA₂ of U.S. Patent No. 5,322,776, some cells contain
10 calcium independent phospholipase A₂/B enzymes. For example, such enzymes have been identified in rat, rabbit, canine and human heart tissue (Gross, TCM, 1991, 2, 115; Zupan et al., J. Med. Chem., 1993, 36, 95; Hazen et al., J. Clin. Invest., 1993, 91, 2513; Lehman et al., J. Biol. Chem., 1993, 268, 20713; Zupan et al., J. Biol. Chem., 1992, 267, 8707; Hazen et al., J. Biol. Chem., 1991, 266,
15 14526; Loeb et al., J. Biol. Chem., 1986, 261, 10467; Wolf et al., J. Biol. Chem., 1985, 260, 7295; Hazen et al., Meth. Enzymol., 1991, 197, 400; Hazen et al., J. Biol. Chem., 1990, 265, 10622; Hazen et al., J. Biol. Chem., 1993, 268, 9892; Ford et al., J. Clin. Invest., 1991, 88, 331; Hazen et al., J. Biol. Chem., 1991, 266, 5629; Hazen et al., Circulation Res., 1992, 70, 486; Hazen et al., J.
20 Biol. Chem., 1991, 266, 7227; Zupan et al., FEBS, 1991, 284, 27), as well as rat and human pancreatic islet cells (Ramanadham et al., Biochemistry, 1993, 32, 337; Gross et al., Biochemistry, 1993, 32, 327), in the macrophage-like cell line, P388D₁ (Ulevitch et al., J. Biol. Chem., 1988, 263, 3079; Ackermann et al., J. Biol. Chem., 1994, 269, 9227; Ross et al., Arch. Biochem. Biophys., 1985, 238,
25 247; Ackermann et al., FASEB Journal, 1993, 7(7), 1237), in various rat tissue cytosols (Nijssen et al., Biochim. Biophys. Acta, 1986, 876, 611; Pierik et al., Biochim. Biophys. Acta, 1988, 962, 345; Aarsman et al., J. Biol. Chem., 1989, 264, 10008), bovine brain (Ueda et al., Biochem. Biophys. Res. Comm., 1993, 195, 1272; Hirashima et al., J. Neurochem., 1992, 59, 708), in yeast
30 (*Saccharomyces cerevisiae*) mitochondria (Yost et al., Biochem. International, 1991, 24, 199), hamster heart cytosol (Cao et al., J. Biol. Chem., 1987, 262, 16027), rabbit lung microsomes (Angle et al., Biochim. Biophys. Acta, 1988, 962,

234) and guinea pig intestinal brush-border membrane (Gassama-Diagne et al., J. Biol. Chem., 1989, 264, 9470).

It is believed that the calcium independent phospholipase A₂/B enzymes may perform important functions in release of arachidonic acid in specific tissues which are characterized by unique membrane phospholipids, by generating lysophospholipid species which are deleterious to membrane integrity or by remodeling of unsaturated species of membrane phospholipids through deacylation/reacylation mechanisms. The activity of such a phospholipase may well be regulated by mechanisms that are different from that of the cPLA₂ of U.S. Patent No. 5,322,776. In addition the activity may be more predominant in certain inflamed tissues over others. Although the enzymatic activity is not dependent on calcium this does not preclude a requirement for calcium *in vivo*, where the activity may be regulated by the interaction of other protein(s) whose function is dependent upon a calcium flux.

15

SUMMARY OF THE INVENTION

In certain embodiments, the present invention provides compositions comprising a purified phospholipase enzyme characterized by (a) activity in the absence of calcium; (b) a molecular weight of 86 kD on SDS-PAGE; and (c) the presence of one or more amino acid sequences selected from the group consisting of NPHSGFR (SEQ ID NO:3), XASXGLNQVNK (SEQ ID NO:4) (X is preferably N or A), YGASPLHXAK (SEQ ID NO:5) (X is preferably W), DNMEMIK (SEQ ID NO:6), GVIYFR (SEQ ID NO:7), MKDEVFR (SEQ ID NO:8), EFGEHTK (SEQ ID NO:9), VMLTGTLSDR (SEQ ID NO:10), XYDAPEVIR (SEQ ID NO:11) (X is preferably N), FNQNINLKPPTQPA (SEQ ID NO:12), XXGAAPTYFRP (SEQ ID NO:13) (X is preferably S), TVFGAK (SEQ ID NO:14), and XWSEMVGIQYFR (SEQ ID NO:15) (X is preferably A), wherein X represents any amino acid residue.

In other embodiments, the invention provides compositions comprising a purified phospholipase enzyme characterized by (a) activity in the absence of calcium; (b) a molecular weight of 86 kD on SDS-PAGE; and (c) the presence of one or more amino acid sequences selected from the group consisting of

YGASPLHXAK, MKDEVFR, EFGEHTK, VMLTGTLSDR, XXGAAPTYFRP and TVFGAK, wherein X represents any amino acid residue.

Certain embodiments provide compositions comprising a purified mammalian calcium independent phospholipase A₂/B enzyme.

5 In other embodiments, the enzyme is further characterized by activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine (preferably a specific activity of about 1 μmol to about 20 μmol per minute per milligram, more preferably a specific activity of about 1 μmol to about 5 μmol per minute per milligram); by a pH optimum of 6; and/or by the absence of
10 stimulation by adenosine triphosphate in the liposome assay.

In other embodiments, the invention provides isolated polynucleotides comprising a nucleotide sequence selected from the group consisting of: (a) the nucleotide sequence of SEQ ID NO:1; (b) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:2; (c) a nucleotide sequence encoding a
15 fragment of the amino acid sequence of SEQ ID NO:2 having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine; (d) a nucleotide sequence capable of hybridizing with the sequence of (a), (b) or (c) which encodes a peptide having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine; and (e) allelic variants of the sequence
20 of (a). Other embodiments provide an isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of: (a) the nucleotide sequence of SEQ ID NO:16; (b) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:17; (c) a nucleotide sequence encoding a fragment of the amino acid sequence of SEQ ID NO:17 having activity in a mixed micelle assay
25 with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine; (d) the nucleotide sequence of SEQ ID NO:18; (e) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:19; (f) a nucleotide sequence encoding a fragment of the amino acid sequence of SEQ ID NO:19 having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine; (g) the nucleotide
30 sequence of SEQ ID NO:20; (h) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:21; (i) a nucleotide sequence encoding a fragment of the amino acid sequence of SEQ ID NO:21 having activity in a mixed micelle assay

with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine; (j) the nucleotide sequence of SEQ ID NO:22; (k) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:23; (l) a nucleotide sequence encoding a fragment of the amino acid sequence of SEQ ID NO:23 having activity in a mixed micelle assay
5 with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine; (m) a nucleotide sequence capable of hybridizing with the sequence of any of (a)-(l) which encodes a peptide having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine; and (n) allelic variants of the sequence of (a), (d), (g) or (j). Expression vectors comprising such polynucleotides and host cells
10 transformed with such vectors are also provided by the present invention. Compositions comprising peptides encoded by such polynucleotides are also provided.

The present invention also provides processes for producing a phospholipase enzyme, said process comprising: (a) establishing a culture of the
15 host cell transformed with a cPLA₂/B encoding polynucleotide in a suitable culture medium; and (b) isolating said enzyme from said culture. Compositions comprising a peptide made according to such processes are also provided.

Certain embodiments of the present invention provide compositions comprising a peptide comprising an amino acid sequence selected from the group
20 consisting of: (a) the amino acid sequence of SEQ ID NO:2; and (b) a fragment of the amino acid sequence of SEQ ID NO:2 having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine.

Other embodiments provide compositions comprising a peptide comprising an amino acid sequence selected from the group consisting of: (a) the amino acid
25 sequence of SEQ ID NO:17; (b) a fragment of the amino acid sequence of SEQ ID NO:17 having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine; (c) the amino acid sequence of SEQ ID NO:19; (d) a fragment of the amino acid sequence of SEQ ID NO:19 having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine;
30 (e) the amino acid sequence of SEQ ID NO:21; (f) a fragment of the amino acid sequence of SEQ ID NO:21 having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine; (g) the amino acid sequence of

SEQ ID NO:23; and (h) a fragment of the amino acid sequence of SEQ ID NO:23 having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine.

The present invention also provides methods for identifying an inhibitor of phospholipase activity, said method comprising: (a) combining a phospholipid, a candidate inhibitor compound, and a composition comprising a phospholipase enzyme peptide; and (b) observing whether said phospholipase enzyme peptide cleaves said phospholipid and releases fatty acid thereby, wherein the peptide composition is one of those described above. Inhibitor of phospholipase activity identified by such methods, pharmaceutical compositions comprising a therapeutically effective amount of such inhibitors and a pharmaceutically acceptable carrier, and methods of reducing inflammation by administering such pharmaceutical compositions to a mammalian subject are also provided.

Polyclonal and monoclonal antibodies to the peptides of the invention are also provided.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1: Fractions containing activity eluted from a Mono P column were examined by reducing SDS-PAGE on a 4-20% gradient gel. Activity of each fraction is shown above the gel and the 86 kD band is indicated on the silver stained gel. Molecular weight markers are indicated.

Fig. 2: Active fractions from a Mono p/Heparin column were combined and further purified on a size exclusion column. Activity eluted in the 250-350 kD size range. Examination of the fractions by SDS-PAGE under reducing conditions on 4-20% gel indicated only one protein band correlated with activity at 86 kD. Molecular weight markers are indicated.

Fig. 3: Active fractions from Mono P eluate and cPLA₂ (0.1-1.0 µg) were analyzed on two 4-20% SDS gels under reducing conditions run in parallel. One gel was silver stained (A) and in the other gel the proteins were transferred to nitrocellulose. the blot was then probed with an anti-cPLA₂ polyclonal antibody and reactive proteins were visualized with the ECL system (Amersham) (B). Molecular weight markers are indicated.

Fig. 4: The activity of the calcium-independent phospholipase eluted from a Mono P/Heparin column and cPLA₂ were compared under conditions which favor each enzyme; pH 7, 10% glycerol in the absence of calcium and pH 9, 70% glycerol in the presence of calcium, respectively.

5 Fig. 5: Activity in the cytosolic extracts of COS cells transfected with: no DNA; plasmid (pED) containing no inserted gene; clone 9 in the antisense orientation; and clones 49, 31 and 9 expressed in pED. The extracts were analyzed under two different assay conditions described for the data presented in Fig. 4.

10 Fig. 6: A comparison of sn-2 fatty acid hydrolysis by activity eluted from a Mono P/Heparin column as a function of the fatty acid substituent at either the sn-1 or sn-2 position and the head group. HAPC, SAPC, PLPC, POPC, PPPC, LYSO and PAPC indicate 1-hexadecyl-2-arachidonyl-, 1-stearoyl-2-arachidonyl-, 1-palmitoyl-2-linoleyl-, 1-palmitoyl-2-oleyl-, 1-palmitoyl-2-palmitoyl-, 1-palmitoyl-, 1-palmitoyl-2-arachidonyl- phosphatidylcholine, respectively. PAPE and SAPI indicate 1-palmitoyl-2-arachidonyl-phosphatidylethanolamine and 1-stearoyl-2-arachidonyl-phosphoinositol, respectively. In all cases the ¹⁴C-labelled fatty acid is in the sn-2 position.

15 Fig. 7: A 4-20% SDS-PAGE of lysates (5x10¹⁰ cpm/lane) of ³⁵S-methionine labelled COS cells transfected with, no DNA, pED (no insert), clone 9 reverse orientation, clones 9, 31 and 49; lanes 1-6, respectively. Molecular weight markers are indicated.

DETAILED DESCRIPTION OF THE INVENTION

25 The present inventors have found surprisingly a calcium independent cytosolic phospholipase enzyme, designated calcium independent cytosolic phospholipase A₂/B or calcium independent cPLA₂/B, purified from the cytosol of Chinese hamster ovary (CHO) cells. The activity was also present in the cytosol of tissues and cell extracts listed in Table I.

Table I

	tissue/cell	mixed micelle pH 7 ($\mu\text{mol}/\text{min}/\text{mg}$)	liposome pH 7 ($\mu\text{mol}/\text{min}/\text{mg}$)
5	rat brain		1-2
	rat heart		0.3-0.5
	bovine brain		0.4
	pig heart	0.8	
	CHO-Dukx	10-20	2-5
10	U937 (ATCC CRL1593)	2	
	FBHE (ATCC CRL1395)	2	
	H9c2 (ATCC Ccl 108)	15	

The enzyme was originally purified by more than 8,000-fold from CHO
 15 cells by sequential chromatography on diethylaminoethane (DEAE), phenyl and
 heparin-toyopearl, followed by chromatofocussing on Mono P (as described further
 in Example 1). In addition the activity could be further purified by size exclusion
 chromatography after the Mono P column. The enzyme eluted from the size
 exclusion chromatography column in the 250-350 kD range, indicating the active
 20 enzyme may consist of a multimeric complex, or may possibly be associated with
 phospholipids.

The calcium independent phospholipase activity correlated with a single
 major protein band of 86 kD on denaturing sodium dodecyl sulfate polyacrylamide
 gel electrophoresis (SDS-PAGE) of active fractions from the Mono P and size
 25 exclusion chromatographic steps; in the latter no protein bands were observed in
 the 250-350 kD range. The specific activity of the enzyme is about 1 μmol to
 about 20 μmol per minute per milligram based on the abundance of the 86 kD
 band in the most active fractions eluted from the Mono P and size exclusion

columns in the mixed micelle assay (Example 3B). The protein band was not recognized by a polyclonal antibody directed against the calcium dependent cPLA₂ of U.S. Patent No. 5,322,776.

The calcium independent phospholipase of the present invention has a pH
5 optimum of 6; its activity is suppressed by calcium (in all assays) and by triton X-100 (in the assay of Example 3A); and is not stimulated by adenosine triphosphate (ATP) (in the assay of Example 3A). The enzyme is inactivated by high concentration denaturants, e.g. urea above 3M, and by detergents, e.g. CHAPS and octyl glucoside. The calcium-independent phospholipase favors hydrolysis by
10 several fold of unsaturated fatty acids, e.g. linoleyl, oleyl and arachidonyl, at the sn-2 position of a phospholipid compared with palmitoyl. In addition there is a preference for palmitoyl at the sn-1 position over hexadecyl or stearyl for arachidonyl hydrolysis at the sn-2 position. In terms of head group substituents there is a clear preference for inositol over choline or ethanolamine when
15 arachidonyl is being hydrolyzed at the sn-2 position. Further, as with cPLA₂ of U.S. Patent No. 5,322,776, there is a significant lysophospholipase activity, i.e. hydrolysis of palmitoyl at the sn-1 position when there is no fatty acid substituent at the sn-2 position. Finally, hydrolysis of fatty acid substituents in the sn-1 or sn-2 in PAPC were compared where either palmitoyl or arachidonyl were labelled
20 with ¹⁴C. Fatty acids were removed at both positions with the sn-2 position having a higher initial rate of hydrolysis by 2-3 fold. This result may indicate sequential hydrolysis of the arachidonyl substituent followed by rapid cleavage of palmitoyl in the lysophospholipid species, which is suggested by the hydrolysis of the individual lipid species. The similar rates of hydrolysis of fatty acid substituents

at the sn-1 (palmitoyl) or sn-2 (arachidonyl) positions, where the radioactive label is in either position, is indicative of a phospholipase B activity. However, the fatty acid substituent at the sn-2 position clearly influences the PLB activity, not the sn-1 fatty acid, since hydrolysis of 1,2-dipalmitoyl substituted phospholipids is substantially less than for the 1-palmitoyl-2-arachidonyl species. These results can be clarified by studying the hydrolysis rates at each position of isotopically dual labelled phospholipids, e.g. ^3H and ^{14}C containing fatty acids at the sn-1 and sn-2 positions, respectively. Therefore, it is prudent to designate the enzyme as a phospholipase A_2/B .

10 A cDNA encoding the calcium independent $cPLA_2/B$ of the present invention was isolated as described in Example 4. The sequence of the cDNA is reported as SEQ ID NO:1. The amino acid sequence encoded by such cDNA is SEQ ID NO:2. The invention also encompasses allelic variations of the cDNA sequence as set forth in SEQ ID NO:1, that is, naturally-occurring alternative
15 forms of the cDNA of SEQ ID NO: 1 which also encode phospholipase enzymes of the present invention.

Other cDNAs encoding a calcium independent $cPLA_2/B$ of the present invention were isolated from human cDNA sources. Two clones identified as "19a" and "19b" were isolated from a Raji cell DNA library derived from
20 Burkitt's lymphoma (ATCC CCL86, commercially available from Clonetech) using a probe derived from the CHO sequence (a 2.1kb Sall-SmaI fragment). Clones 19a and 19b were deposited with the American Type Culture Collection on November 7, 1995 as accession numbers ATCC 69948 and ATCC 69949. The nucleotide sequences of clones 19a and 19b are reported in SEQ ID NO:16 and

SEQ ID NO:18, respectively. SEQ ID NO:17 and SEQ ID NO:18 report the corresponding amino acid sequences encoded by the coding regions of clones 19a and 19b, respectively. Clones 19a and 19b are both partial clones of the full-length human enzyme.

- 5 SEQ ID NO:20 and SEQ ID NO:22 report the nucleotide sequences of alternative ways in which clones 19a and 19b can be spliced to encode a longer partial clone for the full-length human enzyme. The splice occurs after nucleotide 1225 in SEQ ID NO:20 and after nucleotide 1228 in SEQ ID NO:22. The corresponding spliced amino acid sequences are reported in SEQ ID NO:21 and
- 10 SEQ ID NO:23. Spliced cDNA clones can be made from clones 19a and 19b in accordance with methods known to those skilled in the art.

Full-length clones encoding the human enzyme can be isolated by probing human cDNA libraries containing full-length clones using probes derived from SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20 or SEQ ID NO:22.

- 15 Also included in the invention are isolated DNAs which hybridize to the DNA sequence set forth in SEQ ID NO:1, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20 or SEQ ID NO:22 under stringent (e.g. 4xSSC at 65°C or 50% formamide and 4xSSC at 42°C), or relaxed (4xSSC at 50°C or 30-40% formamide at 42°C) conditions.

- 20 The isolated polynucleotides of the invention may be operably linked to an expression control sequence such as the pMT2 or pED expression vectors disclosed in Kaufman *et al.*, Nucleic Acids Res. 19, 4485-4490 (1991), in order to produce the phospholipase enzyme peptides recombinantly. Many suitable expression control sequences are known in the art. General methods of expressing

recombinant proteins are also known and are exemplified in R. Kaufman, Methods in Enzymology 185, 537-566 (1990). As defined herein "operably linked" means enzymatically or chemically ligated to form a covalent bond between the isolated polynucleotide of the invention and the expression control sequence, in such a way

5 that the phospholipase enzyme peptide is expressed by a host cell which has been transformed (transfected) with the ligated polynucleotide/expression control sequence.

A number of types of cells may act as suitable host cells for expression of the phospholipase enzyme peptide. Suitable host cells are capable of attaching

10 carbohydrate side chains characteristic of functional phospholipase enzyme peptide. Such capability may arise by virtue of the presence of a suitable glycosylating enzyme within the host cell, whether naturally occurring, induced by chemical mutagenesis, or through transfection of the host cell with a suitable expression plasmid containing a polynucleotide encoding the glycosylating enzyme. Host cells

15 include, for example, monkey COS cells, Chinese Hamster Ovary (CHO) cells, human kidney 293 cells, human epidermal A431 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other transformed primate cell lines, normal diploid cells, cell strains derived from in vitro culture of primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, or HaK cells.

20 The phospholipase enzyme peptide may also be produced by operably linking the isolated polynucleotide of the invention to suitable control sequences in one or more insect expression vectors, and employing an insect expression system. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, *e.g.*, Invitrogen, San Diego, California,

U.S.A. (the MaxBac® kit), and such methods are well known in the art, as described in Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987), incorporated herein by reference.

Alternatively, it may be possible to produce the phospholipase enzyme peptide in lower eukaryotes such as yeast or in prokaryotes such as bacteria. Potentially suitable yeast strains include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces* strains, *Candida*, or any yeast strain capable of expressing heterologous proteins. Potentially suitable bacterial strains include *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, or any bacterial strain capable of expressing heterologous proteins. If the phospholipase enzyme peptide is made in yeast or bacteria, it is necessary to attach the appropriate carbohydrates to the appropriate sites on the protein moiety covalently, in order to obtain the glycosylated phospholipase enzyme peptide. Such covalent attachments may be accomplished using known chemical or enzymatic methods.

The phospholipase enzyme peptide of the invention may also be expressed as a product of transgenic animals, e.g., as a component of the milk of transgenic cows, goats, pigs, or sheep which are characterized by somatic or germ cells containing a polynucleotide encoding the phospholipase enzyme peptide.

The phospholipase enzyme peptide of the invention may be prepared by culturing transformed host cells under culture conditions necessary to express a phospholipase enzyme peptide of the present invention. The resulting expressed protein may then be purified from culture medium or cell extracts as described in the examples below.

Alternatively, the phospholipase enzyme peptide of the invention is concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred (e.g., S-Sepharose® columns). The purification of the phospholipase enzyme peptide from culture supernatant may also include one or more column steps over such affinity resins as concanavalin A-agarose, heparin-toyopearl® or Cibacrom blue 3GA Sepharose®; or by hydrophobic interaction chromatography using such resins as phenyl ether, butyl ether, or propyl ether; or by immunoaffinity chromatography.

Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify the phospholipase enzyme peptide. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a substantially homogeneous isolated recombinant protein. The phospholipase enzyme peptide thus purified is substantially free of other mammalian proteins and

is defined in accordance with the present invention as "isolated phospholipase enzyme peptide".

The calcium independent cPLA₂/B of the present invention is distinct from the cPLA₂ of U.S. Patent No. 5,322,776 and from previously-described calcium
5 independent phospholipase A₂ enzymes (such as those described by Gross et al., supra; and Ackermann et al., supra). The enzyme of the present invention differs from the cPLA₂ of the '776 patent in the following ways:

- (1) its activity is not calcium dependent;
- (2) it is more active in 10% glycerol than in 70%
10 glycerol;
- (3) it has a molecular weight of 86 kD, not 110 kD as for cPLA₂;
- (4) it has a pH optimum of 6, not greater than 8 as for cPLA₂;
- 15 (5) it hydrolyzes fatty acids at sn-1 as well as sn-2;
- (6) it binds to heparin, while cPLA₂ does not;
- (7) it elutes from an anion exchange column at 0.1-0.2 M NaCl, while cPLA₂ elutes at 0.3-0.4 M NaCl; and
- (8) it does not bind to anti-cPLA₂ polyclonal antibody.

20 The enzyme of the present invention differs from the calcium independent enzyme of Gross et al. in the following characteristics:

- (1) it has a molecular weight of 86 kD, not 40 kD as for the Gross enzyme;

- (2) it is not homologous at the protein level to rabbit skeletal muscle phosphofructokinase in contrast to the 85 kD putative regulatory protein associated with the 40 kD Gross enzyme;
- 5 (3) hydrolysis at the sn-2 position is favored by an acyl-linked fatty acid at the sn-1 position in contrast to ether-linked fatty acids with the Gross enzyme;
- (4) it does not bind to an ATP column and was not activated by ATP in a liposome assay compared to
- 10 the Gross enzyme; and
- (5) it was active in a mixed micelle assay containing Triton X-100.

The enzyme of the present invention differs from the calcium independent enzyme of Ackermann et al. (the "Dennis enzyme") in the following characteristics:

- 15 (1) it does not bind to an ATP column;
- (2) it binds to an anion exchange column (mono Q), while the Dennis enzyme remains in the unbound fraction;
- (3) it has a molecular weight of 86 kD, not 74 kD as for
- 20 the Dennis enzyme;
- (4) it has substantial lysophospholipase activity and is relatively inactive on phospholipids containing ether-linked fatty acids at the sn-1 position in a liposome assay; and

- (5) it appears to hydrolyze fatty acid substituents at the sn-1 and sn-2 positions of a phospholipid, whereas the Dennis enzyme favors hydrolysis at the sn-2 position.

5 The calcium independent cPLA₂/B of the present invention may be used to screen unknown compounds having anti-inflammatory activity mediated by the various components of the arachidonic acid cascade. Many assays for phospholipase activity are known and may be used with the calcium independent phospholipase A₂/B on the present invention to screen unknown compounds. For
10 example, such an assay may be a mixed micelle assay as described in Example 3. Other known phospholipase activity assays include, without limitation, those disclosed in U.S. Patent No. 5,322,776. These assays may be performed manually or may be automated or robotized for faster screening. Methods of automation and robotization are known to those skilled in the art.

15 In one possible screening assay, a first mixture is formed by combining a phospholipase enzyme peptide of the present invention with a phospholipid cleavable by such peptide, and the amount of hydrolysis in the first mixture (B₀) is measured. A second mixture is also formed by combining the peptide, the phospholipid and the compound or agent to be screened, and the amount of
20 hydrolysis in the second mixture (B) is measured. The amounts of hydrolysis in the first and second mixtures are compared, for example, by performing a B/B₀ calculation. A compound or agent is considered to be capable of inhibiting phospholipase activity (i.e., providing anti-inflammatory activity) if a decrease in hydrolysis in the second mixture as compared to the first mixture is observed. The

formulation and optimization of mixtures is within the level of skill in the art. such mixtures may also contain buffers and salts necessary to enhance or to optimize the assay, and additional control assays may be included in the screening assay of the invention.

5 Other uses for the calcium independent cPLA₂/B of the present invention are in the development of monoclonal and polyclonal antibodies. Such antibodies may be generated by employing purified forms of the calcium independent cPLA₂ or immunogenic fragments thereof as an antigen using standard methods for the development of polyclonal and monoclonal antibodies as are known to those skilled
10 in the art. Such polyclonal or monoclonal antibodies are useful as research or diagnostic tools, and further may be used to study phospholipase A₂ activity and inflammatory conditions.

Pharmaceutical compositions containing anti-inflammatory agents (i.e., inhibitors) identified by the screening method of the present invention may be
15 employed to treat, for example, a number of inflammatory conditions such as rheumatoid arthritis, psoriasis, asthma, inflammatory bowel disease and other diseases mediated by increased levels of prostaglandins, leukotriene, or platelet activating factor. Pharmaceutical compositions of the invention comprise a therapeutically effective amount of a calcium independent cPLA₂ inhibitor
20 compound first identified according to the present invention in a mixture with an optional pharmaceutically acceptable carrier. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The term "therapeutically effective amount" means the total amount of each active

component of the method or composition that is sufficient to show a meaningful patient benefit, i.e., healing or amelioration of chronic conditions or increase in rate of healing or amelioration. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a
5 combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously. A therapeutically effective dose of the inhibitor of this invention is contemplated to be in the range of about 0.1 μ g to about 100 mg per kg body weight per application. It is contemplated that the duration of each application of
10 the inhibitor will be in the range of 12 to 24 hours of continuous administration. The characteristics of the carrier or other material will depend on the route of administration.

The amount of inhibitor in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated,
15 and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of inhibitor with which to treat each individual patient. Initially, the attending physician will administer low doses of inhibitor and observe the patient's response. Larger doses of inhibitor may be administered until the optimal therapeutic effect is obtained for the patient, and at
20 that point the dosage is not increased further.

Administration is preferably intravenous, but other known methods of administration for anti-inflammatory agents may be used. Administration of the anti-inflammatory compounds identified by the method of the invention can be carried out in a variety of conventional ways. For example, for topical

administration, the anti-inflammatory compound of the invention will be in the form of a pyrogen-free, dermatologically acceptable liquid or semi-solid formulation such as an ointment, cream, lotion, foam or gel. The preparation of such topically applied formulations is within the skill in the art. Gel formulation
5 should contain, in addition to the anti-inflammatory compound, about 2 to about 5% W/W of a gelling agent. The gelling agent may also function to stabilize the active ingredient and preferably should be water soluble. The formulation should also contain about 2% W/V of a bactericidal agent and a buffering agent. Exemplary gels include ethyl, methyl, and propyl celluloses. Preferred gels
10 include carboxypolymethylene such as Carbopol (934P; B.F. Goodrich), hydroxypropyl methylcellulose phthalates such as Methocel (K100M premium; Merril Dow), cellulose gums such as Blanose (7HF; Aqualon, U.K.), xanthan gums such as Keltrol (TF; Kelco International), hydroxyethyl cellulose oxides such as Polyox (WSR 303; Union Carbide), propylene glycols, polyethylene glycols and
15 mixtures thereof. If Carbopol is used, a neutralizing agent, such as NaOH, is also required in order to maintain pH in the desired range of about 7 to about 8 and most desirably at about 7.5. Exemplary preferred bactericidal agents include steryl alcohols, especially benzyl alcohol. The buffering agent can be any of those already known in the art as useful in preparing medicinal formulations, for
20 example 20 mM phosphate buffer, pH 7.5.

Cutaneous or subcutaneous injection may also be employed and in that case the anti-inflammatory compound of the invention will be in the form of pyrogen-free, parenterally acceptable aqueous solutions. The preparation of such

parenterally acceptable solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art.

Intravenous injection may be employed, wherein the anti-inflammatory compound of the invention will be in the form of pyrogen-free, parenterally acceptable aqueous solutions. A preferred pharmaceutical composition for intravenous injection should contain, in addition to the anti-inflammatory compound, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition according to the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additive known to those of skill in the art.

The amount of anti-inflammatory compound in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of anti-inflammatory compound with which to treat each individual patient.

Anti-inflammatory compounds identified using the method of the present invention may be administered alone or in combination with other anti-inflammation agents and therapies.

Example 1PURIFICATION OF CALCIUM INDEPENDENT cPLA₂

A) Preparation of CHO-Dukx cytosolic fraction:

5 CHO cells, approximately 5×10^{11} cells from a 250L culture, were concentrated by centrifugation and rinsed once with phosphate-buffered saline and reconcentrated. the cell slurry was frozen in liquid nitrogen and stored at -80°C at 4×10^{11} cells/kg of pellet. The CHO pellets were processed in 0.5kg batches by thawing the cells in 1.2L of 20mM imidazol pH 7.5, 0.25M sucrose, 2mM EDTA,
10 2mM EGTA, $1\mu\text{g/ml}$ leupeptin, $5\mu\text{g/ml}$ aprotinin, 5mM DTT and 1mM PMSF ("Extraction Buffer"). The cells were transferred to a Parr bomb at 4°C and pressurized at 600psi for 5 minutes and lysed by releasing the pressure. The supernatant was centrifuged at $10,000 \times g$ for 30 minutes and subsequently at $100,000 \times g$ for 60 minutes.

15

B) DEAE anion exchange chromatography:

The cytosolic fraction (10gm protein) was diluted to 5mg/ml with 20mM imidazol pH 7.5, 5mM DTT, 1mM EDTA and 1mM EGTA (Buffer A) and applied to a 1L column of DEAE toyopearl equilibrated in buffer A at 16ml/min.
20 The column was washed to background absorbance (A_{280}) with buffer A and developed with a gradient of 0-0.5M NaCl in buffer A over 240 minutes with one minute fractions. The first activity peak at 100-150mM NaCl was collected.

C) Hydrophobic interaction and heparin toyopearl chromatography:

The DEAE fractions (4gm of protein at 3mg/ml) were made 0.5M in ammonium sulfate and applied at 10ml/min to a 300ml phenyl toyopearl column equilibrated in buffer A containing 0.5M ammonium sulfate. The column was washed to background absorbance (A_{280}). The column was then developed with a
5 gradient of 0.5-0.2M (15 minutes) then 0.2-0.0 M ammonium sulfate (85 minutes). The column was then connected in tandem to a 10ml heparin column equilibrated in buffer A and elution was continued for 18 hours at 1.5ml/min with buffer A. The phenyl column was disconnected and the activity was eluted from the heparin column by applying 0.5M NaCl in buffer A at 2ml/min.

10

D) Chromatofocussing Chromatography:

A portion of the above active fractions (16mg) was dialyzed exhaustively against 20mM Bis-Tris pH 7, 10% glycerol, 1M urea and 5mM DTT and applied at 0.5ml/min to a Mono P 5/20 column equilibrated with the same buffer. The
15 column was washed with the same buffer to background absorbance (A_{280}) and a pH gradient was established by applying 10% polybuffer 74 pH 5, 10% glycerol, 1M urea and 5mM DTT.

The relative purification of the enzyme of the present invention at each step of the foregoing purification scheme is summarized in Table II.

Table II

Step	Protein (mg)	Activity (u ^{**})	Specific Activity (u/mg)	Fold Purification	Yield (%)
cytosolic extract [*]	126,000	2050	0.016	--	--
DEAE	16,000	1264	0.079	5	60
phenyl/heparin	193	90	0.46	30	4.5
Mono P	0.1-0.2	14	140	8,000	0.7

^{*}Extract from 3.5 kg of frozen CHO cell pellet

^{**}1 unit is defined as the amount of activity that releases 1 nmol of arachidonic acid per minute

The phospholipase can be further purified by the following steps:

E) Heparin chromatography:

The sample from (D) above is applied at 0.5ml/min onto a heparin column (maximum capacity 10mg protein/ml of resin) equilibrated in buffer A. The activity is eluted by 0.4M NaCl in buffer A.

F) Size exclusion chromatography:

The active fractions from the heparin column are applied to two TSK G3000SW_{XL} columns (7.8mm x 30cm) linked in tandem equilibrated with 150mM NaCl in buffer A at 0.3ml/min. Phospholipase activity elutes in the 250-350 kD size range.

Recombinant enzyme may also be purified in accordance with this example.

Example 2

AMINO ACID SEQUENCING

A portion (63 μ g total protein) of the Mono P active fractions was concentrated on a heparin column, as described above. The sample, 0.36ml was
5 mixed with an equal volume of buffer A and 10% SDS, 10 μ l and concentrated to 40 μ l on an Amicon-30 microconcentrator. The sample was diluted with buffer A, 100 μ l, concentrated to 60 μ l and diluted with Laemmli buffer (2x), 40 μ l. The solution was boiled for 5 minutes and loaded in three aliquots on a 4-20% gradient SDS-PAGE mini gel. The sample was electrophoresed for two hours at 120v,
10 stained for 20 minutes in 0.2% Blue R-250, 20% methanol and 0.5% acetic acid and destained in 30% methanol (Rosenfeld et. al. Anal. Biochem. 203, pp. 173-179, 1992). Briefly, the protein bands corresponding to the phospholipase were excised from the gel with a razor blade and washed with 4 150 μ l aliquots of 200 mM NH_4HCO_3 , 50% acetonitrile, for a total of 2 hours. The gel pieces were
15 allowed to air dry for approximately 5 minutes, then partially rehydrated with 1 μ l of 200 mM NH_4HCO_3 , 0.02% Tween 20 (Pierce) and 2 μ l of 0.25 μ g/ μ l trypsin (Promega). Gel slices were placed into the bottom of 500 μ l mini-Eppendorf tubes, covered with 30 μ l 200 mM NH_4HCO_3 , and incubated at 37 C for 15 hours. After 1-2 minutes of
20 centrifugation in an Eppendorf microfuge, the supernatants were removed and saved. Peptides in the gel slices were extracted by agitation for a total of 40 minutes with 2 100 μ l aliquots of 60% acetonitrile, 0.1% TFA. The extracts were combined with the previous supernatant. The volume was reduced by lyophilization to about 150 μ l, and then the sample was diluted with 750 μ l 0.1% TFA. Peptide

maps were run on an ABI 130A Separation System HPLC and an ABI 30 X 2.1 mm RP-300 column. The gradient used was as follows: 0-13.5 minutes 0% B, 13.5-63.5 minutes 0-100% B and 63.5-68.5 minutes 100% B, where A is 0.1% TFA and B is 0.085% TFA, 70% acetonitrile. Peptides were then sequenced on
5 an ABI 470A gas-phase sequencer.

Example 3

PHOPHOLIPASE ASSAYS

1. sn-2 Hydrolysis Assays

10 A) Liposome: The lipid, e.g. 1-palmitoyl-2-[¹⁴C]arachidonyl-sn-glycero-3-phosphocholine (PAPC), 55 mCi/mmol, was dried under a stream of nitrogen and solubilized in ethanol. The assay buffer contained 100mM Tris-HCl pH 7, 4mM EDTA, 4mM EGTA, 10% glycerol and 25μM of labelled PAPC, where the volume of ethanol added was no more than 10% of the final assay volume. The
15 reaction was incubated for 30 minutes at 37°C and quenched by the addition of two volumes of heptane:isopropanol:0.5M sulfuric acid (105:20:1 v/v). Half of the organic was applied to a disposable silica gel column in a vacuum manifold positioned over a scintillation vial, and the free arachidonic was eluted by the addition of ethyl ether (1ml). The level of radioactivity was measured by liquid
20 scintillation.

Variations on this assay replace EDTA and EGTA with 10mM CaCl₂.

B) Mixed Micelle Basic: The lipid was dried down as in (A) and to this was added the assay buffer consisting of 80mM glycine pH 9, 5mM CaCl₂ or

5mM EDTA. 10% or 70% glycerol and 200 μ M triton X-100. The mixture was then sonicated for 30-60 seconds at 4°C to form mixed micelles.

C) Mixed Micelle Neutral: As for (B) except 100mM Tris-HCl pH 7 was used instead of glycine as the buffer.

5

2. sn-1 Hydrolysis Assays

Sn-1 hydrolysis assays are performed as described above for sn-1 hydrolysis, but using phospholipids labelled at the sn-1 substituent, e.g. 1-[¹⁴C]-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine.

10

Example 4

CLONING OF CALCIUM INDEPENDENT cPLA₂/B

A) cDNA Library Construction

Total RNA was first prepared from 2 x 10⁸ CHO-DUX cells using the
15 RNAgents total RNA kit (Promega, Madison, Wisconsin) and further purified using the PolyA Tract mRNA Isolation System (Promega) to yield 13.2 μ g polyA + mRNA. Double stranded cDNA was prepared by the Superscript Choice System (Gibco/BRL, Gaithersburg, Maryland) starting with 2 μ g of CHO-DUX mRNA and using oligo dT primer. The cDNA was modified at both ends by addition of
20 an EcoRI adapter/linker provided by the kit. These fragments were then ligated into the predigested lambda ZAPII/EcoRI vector, and packaged into phage particles with Gigapack Gold packaging extracts (Stratagene, La Jolla, California).

B) Oligonucleotide Probe Design

Several of the peptide sequences determined for the purified calcium independent PLA₂/B were selected to design oligonucleotide probes. The amino acid sequence from amino acid 361 to 367 of SEQ ID NO:2 was used to design
5 two degenerate oligonucleotide pools of 17 residues each. Pool 1 is 8-fold degenerate representing the sense strand for amino acids 361 to 366 of SEQ ID NO:2, and pool 2 is 12-fold degenerate representing the antisense strand for amino acids 362-367 of SEQ ID NO:2. Two other degenerate pools were also made from other sequences. Pool 3 is 32-fold degenerate and represents the sense strand
10 for amino acids 490 to 495 of SEQ ID NO:2, and pool 4 is 64-fold degenerate representing the antisense strand for amino acids 513 to 518 of SEQ ID NO:2.

C) Library Screening

Approximately 400,000 recombinant bacteriophage from the CHO-DUX
15 cDNA library were plated and duplicate nitrocellulose filters were prepared. One set of filters was hybridized with pool 1 and the other with pool 2 using tetramethylammonium chloride buffer conditions (Jacobs et al., Nature, 1985, 313, 806). Twelve positive bacteriophages were identified and plated for further analysis. Three sets of nitrocellulose filters were prepared from this plating and
20 hybridized with pools 2, 3 and 4, to represent the three peptide sequences from which probes were designed. Several clones were positive for all three pools. Individual bacteriophage plaques were eluted and ampicillin resistant plasmid colonies were prepared following the manufacturer's protocols (Stratagene). Plasmid DNA was prepared for clones 9, 17, 31 and 49, and restriction digests

revealed 3.0 kb inserts. Analysis of a portion of the DNA sequence in these clones confirmed that they contained several cPLA₂/B peptide sequences and represented the complete coding region of the gene. Clone 9 was selected for complete DNA sequence determination. The sequence of clone 9 is reported as

5 SEQ ID NO:1.

Clone 9 was deposited with ATCC on July 27, 1994 as accession number 69669.

Example 5

10 EXPRESSION OF RECOMBINANT cPLA₂/B

A) Expression in COS Cells

Clone 9 from Example 4 was excised inserted into a Sall site that was engineered into the EcoRI site of the COS expression vector, PMT-2, a beta lactamase derivative of p91023 (Wong et al., Science, 1985, 228, 810). 8 µg of
15 plasmid DNA was then transfected into 1 x 10⁶ COS cells in a 10 cm dish by the DEAE dextran protocol (Sompayrac et al., Proc. Natl. Acad. Sci. USA, 1981, 78, 7575) with the addition of a 0.1 mM chloroquine to the transfection medium, followed by incubation for 3 hours at 37°C. The cells were grown in conventional media (DME, 10% fetal calf serum). At 40-48 hours post-transfection the cells
20 were washed twice and then incubated at 37°C in PBS, 1 mM EDTA (5 ml). The cells were then collected by centrifugation, resuspended in Extraction Buffer (0.5 ml), and lysed by 20 strokes in a Dounce at 4°C. The lysate was clarified by centrifugation and 10-50 µl of the cytosolic fraction was assayed in the neutral and pH 9 mixed micelle assays.

In a further experiment, COS cells were transiently transfected according to established procedures (Kaufman et al.). After 40-48 hours post-transfection the cells were labelled with ^{35}S -methionine, 200 μCi per 10 cm plate, for one hour and the cells were lysed in NP-40 lysis buffer (Kaufman et al.). The cell lysates
5 were analyzed by SDS-PAGE on a 4-20% reducing gel where equal counts were loaded per lane. There was an additional protein band at 84-86 kD in the lysates from cells transfected with clones 9, 31 and 49, but not in controls (see Fig. 7).

B) Expression in CHO Cells

10 A single plasmid bearing both the cPLA₂/B encoding sequence and a DHFR gene, or two separate plasmids bearing such sequences, are introduced into DHFR-deficient CHO cells (such as Dukx-BII) by calcium phosphate coprecipitation and transfection. DHFR expressing transformants are selected for growth in alpha media with dialyzed fetal calf serum. Transformants are checked for expression
15 of recombinant enzyme by bioassay, immunoassay or RNA blotting and positive pools are subsequently selected for amplification by growth in increasing concentrations of methotrexate (MTX) (sequential steps in 0.02, 0.2, 1.0 and 5 μM MTX) as described in Kaufman et al., Mol. Cell Biol., 1983, 5, 1750. The amplified lines are cloned and recombinant enzyme expression is monitored by the
20 mixed micelle assay. Recombinant enzyme expression is expected to increase with increasing levels of MTX resistance.

Example 6

MUTAGENESIS OF SERINE RESIDUES

Ser252 and Ser465 of the murine cPLA₂/B amino acid sequence were mutated to alanine residues using the Chamelon Mutagenesis kit (Stratagene) using
5 oligonucleotides CATGGGACCCGCTGGCTTTCC (SEQ ID NO:24) and GGCAGGAACCCGCCACTGGGGGC (SEQ ID NO:25), respectively. PLA₂ activity was abrogated by changing Ser465 to Ala in the lipase consensus sequence (GX_SXGG) surrounding that residue. Although Ser252 is found in a partial lipase motif, mutagenesis did not result in loss of activity. Moreover, Ser465, and the
10 lipase consensus sequence surrounding this residue, are conserved in the human sequence (see amino acids 462-467 of SEQ ID NO:21 and 463-468 of SEQ ID NO:23), while Ser252 is not. On this basis, it is believed that this conserved serine residue is required for activity.

15 Patent and literature references cited herein are incorporated by reference as if fully set forth.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Jones, Simon
Tang, Jim
- (ii) TITLE OF INVENTION: Calcium Independent Phospholipase A2/B
- (iii) NUMBER OF SEQUENCES: 25
- (iv) COMPUTER READABLE FORM:
 (A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 2935 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iii) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 96..2352

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GCGGCCGCGT CGACGAAGTA AGCGGGCGGA GAAGTGCTGA GTAAGCCGAG AGTAAGGGGG      60
CAGGCTGTCC CCCCCCCCCA CCTGCCCCAC GGAGG ATG CAG TTC TTC GGA CGC      113
                               Met Gln Phe Phe Gly Arg
                               1           5

CTT GTC AAC ACC CTC AGT AGT GTC ACC AAC TTG TTC TCA AAC CCA TTC      161
Leu Val Asn Thr Leu Ser Ser Val Thr Asn Leu Phe Ser Asn Pro Phe
          10          15          20

CGG GTG AAG GAG ATA TCT GTG GCT GAC TAT ACC TCA CAT GAA CGT GTT      209
Arg Val Lys Glu Ile Ser Val Ala Asp Tyr Thr Ser His Glu Arg Val
          25          30          35

CGA GAG GAA GGG CAG CTG ATC CTG TTC CAG AAT GCT TCC AAT CGC ACC      257
Arg Glu Glu Gly Gln Leu Ile Leu Phe Gln Asn Ala Ser Asn Arg Thr
          40          45          50

TGG GAC TGC ATC CTG GTC AGC CCT AGG AAC CCA CAT AGT GGC TTC CGA      305
Trp Asp Cys Ile Leu Val Ser Pro Arg Asn Pro His Ser Gly Phe Arg
          55          60          65          70

CTC TTC CAG CTG GAG TCA GAG GCA GAT GCC CTG GTG AAC TTC CAG CAG      353
Leu Phe Gln Leu Glu Ser Glu Ala Asp Ala Leu Val Asn Phe Gln Gln
          75          80          85

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TTC	TCC	TCC	CAG	CTG	CCA	CCC	TTC	TAC	GAG	AGC	TCT	GTG	CAG	GTC	CTG	401
Phe	Ser	Ser	Gln	Leu	Pro	Pro	Phe	Tyr	Glu	Ser	Ser	Val	Gln	Val	Leu	
			90					95					100			
CAT	GTG	GAG	GTG	CTG	CAG	CAC	CTG	TCT	GAC	CTG	ATC	CGA	AGC	CAC	CCC	449
His	Val	Glu	Val	Leu	Gln	His	Leu	Ser	Asp	Leu	Ile	Arg	Ser	His	Pro	
		105					110					115				
AGC	TGG	ACG	GTG	ACA	CAC	CTG	GCG	GTG	GAG	CTT	GGC	ATT	CGG	GAG	TGC	497
Ser	Trp	Thr	Val	Thr	His	Leu	Ala	Val	Glu	Leu	Gly	Ile	Arg	Glu	Cys	
	120					125					130					
TTC	CAC	CAC	AGC	CGC	ATC	ATC	AGC	TGC	GCC	AAC	AGC	ACA	GAG	AAT	GAG	545
Phe	His	His	Ser	Arg	Ile	Ile	Ser	Cys	Ala	Asn	Ser	Thr	Glu	Asn	Glu	
					140					145					150	
GAG	GGC	TGC	ACC	CCA	CTG	CAT	TTG	GCA	TGC	CGC	AAG	GGT	GAC	AGT	GAG	593
Glu	Gly	Cys	Thr	Pro	Leu	His	Leu	Ala	Cys	Arg	Lys	Gly	Asp	Ser	Glu	
				155					160					165		
ATC	CTG	GTG	GAG	TTG	GTA	CAG	TAC	TGC	CAT	GCC	CAA	ATG	GAT	GTC	ACT	641
Ile	Leu	Val	Glu	Leu	Val	Gln	Tyr	Cys	His	Ala	Gln	Met	Asp	Val	Thr	
			170					175					180			
GAC	AAC	AAA	GGA	GAG	ACG	GCC	TTC	CAT	TAC	GCT	GTA	CAA	GGG	GAC	AAT	689
Asp	Asn	Lys	Gly	Glu	Thr	Ala	Phe	His	Tyr	Ala	Val	Gln	Gly	Asp	Asn	
		185					190					195				
TCC	CAG	GTG	CTG	CAG	CTC	CTA	GGA	AAG	AAC	GCC	TCA	GCT	GGC	CTG	AAC	737
Ser	Gln	Val	Leu	Gln	Leu	Leu	Gly	Lys	Asn	Ala	Ser	Ala	Gly	Leu	Asn	
		200				205					210					
CAG	GTG	AAC	AAA	CAA	GGG	CTA	ACT	CCA	CTG	CAC	CTG	GCC	TGC	CAG	ATG	785
Gln	Val	Asn	Lys	Gln	Gly	Leu	Thr	Pro	Leu	His	Leu	Ala	Cys	Gln	Met	
		215			220					225					230	
GGG	AAG	CAG	GAG	ATG	GTA	CGC	GTC	CTG	CTG	CTT	TGC	AAT	GCC	CGC	TGC	833
Gly	Lys	Gln	Glu	Met	Val	Arg	Val	Leu	Leu	Cys	Asn	Ala	Arg	Cys		
				235				240					245			
AAC	GTC	ATG	GGA	CCC	AGT	GGC	TTT	CCC	ATC	CAC	ACA	GCC	ATG	AAG	TTC	881
Asn	Val	Met	Gly	Pro	Ser	Gly	Phe	Pro	Ile	His	Thr	Ala	Met	Lys	Phe	
			250					255					260			
TCC	CAG	AAG	GGG	TGT	GCT	GAA	ATG	ATT	ATC	AGC	ATG	GAC	AGC	AGC	CAG	929
Ser	Gln	Lys	Gly	Cys	Ala	Glu	Met	Ile	Ile	Ser	Met	Asp	Ser	Ser	Gln	
		265					270					275				
ATC	CAC	AGC	AAG	GAT	CCT	CGC	TAT	GGA	GCC	AGC	CCG	CTC	CAC	TGG	GCC	977
Ile	His	Ser	Lys	Asp	Pro	Arg	Tyr	Gly	Ala	Ser	Pro	Leu	His	Trp	Ala	
		280				285					290					
AAG	AAT	GCC	GAG	ATG	GCC	CGG	ATG	CTG	CTG	AAG	CGG	GGA	TGT	GAT	GTG	1025
Lys	Asn	Ala	Glu	Met	Ala	Arg	Met	Leu	Leu	Lys	Arg	Gly	Cys	Asp	Val	
		295			300					305					310	
GAC	AGC	ACA	AGC	GCT	GCG	GGG	AAC	ACA	GCC	CTG	CAT	GTG	GCA	GTG	ATG	1073
Asp	Ser	Thr	Ser	Ala	Ala	Gly	Asn	Thr	Ala	Leu	His	Val	Ala	Val	Met	
				315					320				325			
CGG	AAC	CGC	TTT	GAC	TGC	GTC	ATG	GTG	CTG	CTG	ACC	TAC	GGG	GCC	AAC	1121
Arg	Asn	Arg	Phe	Asp	Cys	Val	Met	Val	Leu	Leu	Thr	Tyr	Gly	Ala	Asn	
			330					335					340			
GCA	GGC	ACC	CCA	GGG	GAG	CAT	GGG	AAC	ACG	CCG	CTG	CAC	CTG	GCC	ATC	1169
Ala	Gly	Thr	Pro	Gly	Glu	His	Gly	Asn	Thr	Pro	Leu	His	Leu	Ala	Ile	
		345					350					355				

TCG AAA GAT AAC ATG GAG ATG ATC AAA GCC CTC ATT GTA TTT GGG GCA Ser Lys Asp Asn Met Glu Met Ile Lys Ala Leu Ile Val Phe Gly Ala 360 365 370	1217
GAA GTG GAT ACC CCA AAT GAC TTT GGG GAG ACT CCT GCC TTC ATG GCC Glu Val Asp Thr Pro Asn Asp Phe Gly Glu Thr Pro Ala Phe Met Ala 375 380 385 390	1265
TCC AAG ATC AGC AAA CAG CTT CAG GAC CTC ATG CCC ATC TCC CGA GCC Ser Lys Ile Ser Lys Gln Leu Gln Asp Leu Met Pro Ile Ser Arg Ala 395 400 405	1313
CGG AAG CCA GCA TTC ATC CTG AGC TCC ATG AGG GAT GAG AAG CGA ATC Arg Lys Pro Ala Phe Ile Leu Ser Ser Met Arg Asp Glu Lys Arg Ile 410 415 420	1361
CAT GAT CAC CTG CTC TGC CTG GAC GGA GGG GGC GTG AAA GGC CTG GTC His Asp His Leu Leu Cys Leu Asp Gly Gly Gly Val Lys Gly Leu Val 425 430 435	1409
ATC ATC CAA CTC CTC ATT GCC ATC GAG AAG GCC TCA GGT GTG GCC ACC Ile Ile Gln Leu Leu Ile Ala Ile Glu Lys Ala Ser Gly Val Ala Thr 440 445 450	1457
AAG GAC CTC TTC GAC TGG GTG GCA GGA ACC AGC ACT GGG GGC ATC CTG Lys Asp Leu Phe Asp Trp Val Ala Gly Thr Ser Thr Gly Gly Ile Leu 455 460 465 470	1505
GCC CTG GCC ATT CTG CAC AGT AAG TCC ATG GCC TAT ATG CGT GGT GTG Ala Leu Ala Ile Leu His Ser Lys Ser Met Ala Tyr Met Arg Gly Val 475 480 485	1553
TAC TTC CGT ATG AAA GAT GAG GTG TTT CGG GGC TCA CGG CCC TAT GAG Tyr Phe Arg Met Lys Asp Glu Val Phe Arg Gly Ser Arg Pro Tyr Glu 490 495 500	1601
TCT GGA CCC CTG GAG GAG TTC CTG AAG CGG GAG TTT GGG GAG CAC ACC Ser Gly Pro Leu Glu Glu Phe Leu Lys Arg Glu Phe Gly Glu His Thr 505 510 515	1649
AAG ATG ACA GAT GTC AAA AAA CCC AAG GTG ATG CTC ACA GGG ACA CTG Lys Met Thr Asp Val Lys Lys Pro Lys Val Met Leu Thr Gly Thr Leu 520 525 530	1697
TCT GAC CGG CAG CCA GCA GAG CTC CAC CTG TTC CGC AAT TAC GAT GCT Ser Asp Arg Gln Pro Ala Glu Leu His Leu Phe Arg Asn Tyr Asp Ala 535 540 545 550	1745
CCA GAG GTC ATT CGG GAA CCT CGC TTC AAC CAA AAC ATT AAC CTG AAG Pro Glu Val Ile Arg Glu Pro Arg Phe Asn Gln Asn Ile Asn Leu Lys 555 560 565	1793
CCG CCA ACT CAG CCT GCA GAC CAA CTG GTA TGG CGA GCA GCC CGG AGC Pro Pro Thr Gln Pro Ala Asp Gln Leu Val Trp Arg Ala Ala Arg Ser 570 575 580	1841
AGT GGG GCA GCC CCA ACC TAC TTC CGG CCC AAT GGA CGT TTC CTG GAT Ser Gly Ala Ala Pro Thr Tyr Phe Arg Pro Asn Gly Arg Phe Leu Asp 585 590 595	1889
GGT GGG CTG CTG GCC AAC AAC CCC ACA CTA GAT GCC ATG ACT GAA ATC Gly Gly Leu Leu Ala Asn Asn Pro Thr Leu Asp Ala Met Thr Glu Ile 600 605 610	1937
CAT GAA TAC AAT CAG GAC ATG ATC CGC AAG GGC CAA GGC AAC AAG GTG His Glu Tyr Asn Gln Asp Met Ile Arg Lys Gly Gln Gly Asn Lys Val 615 620 625 630	1985

AAG AAA CTC TCC ATA GTC GTC TCT CTG GGG ACA GGA AGG TCC CCT CAA	2033
Lys Lys Leu Ser Ile Val Val Ser Leu Gly Thr Gly Arg Ser Pro Gln	
635 640 645	
GTG CCC GTA ACC TGT GTA GAT GTC TTC CGC CCC AGC AAC CCC TGG GAA	2081
Val Pro Val Thr Cys Val Asp Val Phe Arg Pro Ser Asn Pro Trp Glu	
650 655 660	
CTG GCT AAG ACT GTT TTT GGA GCC AAG GAA CTG GGC AAG ATG GTG GTA	2129
Leu Ala Lys Thr Val Phe Gly Ala Lys Glu Leu Gly Lys Met Val Val	
665 670 675	
GAC TGT TGC ACA GAT CCA GAT GGT CGG GCT GTG GAC CGG GCC CGG GCC	2177
Asp Cys Cys Thr Asp Pro Asp Gly Arg Ala Val Asp Arg Ala Arg Ala	
680 685 690	
TGG AGC GAG ATG GTT GGC ATC CAG TAC TTC AGA CTG AAC CCC CAA CTA	2225
Trp Ser Glu Met Val Gly Ile Gln Tyr Phe Arg Leu Asn Pro Gln Leu	
695 700 705 710	
GGA TCA GAC ATC ATG CTG GAT GAG GTC AAT GAT GCA GTG CTG GTT AAT	2273
Gly Ser Asp Ile Met Leu Asp Glu Val Asn Asp Ala Val Leu Val Asn	
715 720 725	
GCC CTC TGG GAG ACA GAA GTC TAC ATC TAT GAG CAC CGG GAG GAG TTC	2321
Ala Leu Trp Glu Thr Glu Val Tyr Ile Tyr Glu His Arg Glu Glu Phe	
730 735 740	
CAG AAG CTT GTC CAA ATG CTG CTG TCG CCC T GAGCTCCAGG CCCTGCTGGC	2372
Gln Lys Leu Val Gln Met Leu Leu Ser Pro	
745 750	
AGGGGTGCGC CAGGCTACCC AGCACACTGG GGGCCAAGCT GGGCCAGGCG GCTGTGTCTA	2432
CCTGAGGACT GGGGCTCAGA GCACAAACAG GTTCCCACAA GGCACCTCTC CTGACCCATC	2492
TGCACTTTGC CACTCTAGGC TGAAAGCCCA GAGTTCCCTT CAGCCCCTTT ATGTGACTGT	2552
GAAGGACAAC TGGCTCCATC AACTGCCCTA AATATCAGTG AGATCAACAC TAAGGTGTCC	2612
AGTGTACCCA GAGGGTTCTT CCAGGTCCA TGGCCACCAA AGCCCACCCC TTCTTCCAC	2672
TTCTGAAGT CAGTGTCTAC AGAAATGGAG TTCCACCCCA TCATCAGGTG AAATCCAGGC	2732
TATTGAAATC CAGTCTGTTC GACTTTGCCC CTCTGCACCT GCCAATCACC CCACCCCTGC	2792
AGCCACCCCA CCTTAAGAGT CCTCCAGCT CTCAAAGGTC AATCCTGTGC ATGTACTCTT	2852
CTCTGGAAGG AGAGTGGGGA GGGGTTCAAG GCCACCTCAA CTGTGAAATA AATGGGTCTA	2912
GACTCAAAAA AAAAAAGTCG ACG	2935

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 752 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Gln Phe Phe Gly Arg Leu Val Asn Thr Leu Ser Ser Val Thr Asn
 1 5 10 15

Leu Phe Ser Asn Pro Phe Arg Val Lys Glu Ile Ser Val Ala Asp Tyr
 20 25 30
 Thr Ser His Glu Arg Val Arg Glu Glu Gly Gln Leu Ile Leu Phe Gln
 35 40 45
 Asn Ala Ser Asn Arg Thr Trp Asp Cys Ile Leu Val Ser Pro Arg Asn
 50 55 60
 Pro His Ser Gly Phe Arg Leu Phe Gln Leu Glu Ser Glu Ala Asp Ala
 65 70 75 80
 Leu Val Asn Phe Gln Gln Phe Ser Ser Gln Leu Pro Pro Phe Tyr Glu
 85 90 95
 Ser Ser Val Gln Val Leu His Val Glu Val Leu Gln His Leu Ser Asp
 100 105 110
 Leu Ile Arg Ser His Pro Ser Trp Thr Val Thr His Leu Ala Val Glu
 115 120 125
 Leu Gly Ile Arg Glu Cys Phe His His Ser Arg Ile Ile Ser Cys Ala
 130 135 140
 Asn Ser Thr Glu Asn Glu Glu Gly Cys Thr Pro Leu His Leu Ala Cys
 145 150 155 160
 Arg Lys Gly Asp Ser Glu Ile Leu Val Glu Leu Val Gln Tyr Cys His
 165 170 175
 Ala Gln Met Asp Val Thr Asp Asn Lys Gly Glu Thr Ala Phe His Tyr
 180 185 190
 Ala Val Gln Gly Asp Asn Ser Gln Val Leu Gln Leu Leu Gly Lys Asn
 195 200 205
 Ala Ser Ala Gly Leu Asn Gln Val Asn Lys Gln Gly Leu Thr Pro Leu
 210 215 220
 His Leu Ala Cys Gln Met Gly Lys Gln Glu Met Val Arg Val Leu Leu
 225 230 235 240
 Leu Cys Asn Ala Arg Cys Asn Val Met Gly Pro Ser Gly Phe Pro Ile
 245 250 255
 His Thr Ala Met Lys Phe Ser Gln Lys Gly Cys Ala Glu Met Ile Ile
 260 265 270
 Ser Met Asp Ser Ser Gln Ile His Ser Lys Asp Pro Arg Tyr Gly Ala
 275 280 285
 Ser Pro Leu His Trp Ala Lys Asn Ala Glu Met Ala Arg Met Leu Leu
 290 295 300
 Lys Arg Gly Cys Asp Val Asp Ser Thr Ser Ala Ala Gly Asn Thr Ala
 305 310 315 320
 Leu His Val Ala Val Met Arg Asn Arg Phe Asp Cys Val Met Val Leu
 325 330 335
 Leu Thr Tyr Gly Ala Asn Ala Gly Thr Pro Gly Glu His Gly Asn Thr
 340 345 350
 Pro Leu His Leu Ala Ile Ser Lys Asp Asn Met Glu Met Ile Lys Ala
 355 360 365

Leu Ile Val Phe Gly Ala Glu Val Asp Thr Pro Asn Asp Phe Gly Glu
 370 375 380
 Thr Pro Ala Phe Met Ala Ser Lys Ile Ser Lys Gln Leu Gln Asp Leu
 385 390 395 400
 Met Pro Ile Ser Arg Ala Arg Lys Pro Ala Phe Ile Leu Ser Ser Met
 405 410 415
 Arg Asp Glu Lys Arg Ile His Asp His Leu Leu Cys Leu Asp Gly Gly
 420 425 430
 Gly Val Lys Gly Leu Val Ile Ile Gln Leu Leu Ile Ala Ile Glu Lys
 435 440 445
 Ala Ser Gly Val Ala Thr Lys Asp Leu Phe Asp Trp Val Ala Gly Thr
 450 455 460
 Ser Thr Gly Gly Ile Leu Ala Leu Ala Ile Leu His Ser Lys Ser Met
 465 470 475 480
 Ala Tyr Met Arg Gly Val Tyr Phe Arg Met Lys Asp Glu Val Phe Arg
 485 490 495
 Gly Ser Arg Pro Tyr Glu Ser Gly Pro Leu Glu Glu Phe Leu Lys Arg
 500 505 510
 Glu Phe Gly Glu His Thr Lys Met Thr Asp Val Lys Lys Pro Lys Val
 515 520 525
 Met Leu Thr Gly Thr Leu Ser Asp Arg Gln Pro Ala Glu Leu His Leu
 530 535 540
 Phe Arg Asn Tyr Asp Ala Pro Glu Val Ile Arg Glu Pro Arg Phe Asn
 545 550 555 560
 Gln Asn Ile Asn Leu Lys Pro Pro Thr Gln Pro Ala Asp Gln Leu Val
 565 570 575
 Trp Arg Ala Ala Arg Ser Ser Gly Ala Ala Pro Thr Tyr Phe Arg Pro
 580 585 590
 Asn Gly Arg Phe Leu Asp Gly Gly Leu Leu Ala Asn Asn Pro Thr Leu
 595 600 605
 Asp Ala Met Thr Glu Ile His Glu Tyr Asn Gln Asp Met Ile Arg Lys
 610 615 620
 Gly Gln Gly Asn Lys Val Lys Lys Leu Ser Ile Val Val Ser Leu Gly
 625 630 635 640
 Thr Gly Arg Ser Pro Gln Val Pro Val Thr Cys Val Asp Val Phe Arg
 645 650 655
 Pro Ser Asn Pro Trp Glu Leu Ala Lys Thr Val Phe Gly Ala Lys Glu
 660 665 670
 Leu Gly Lys Met Val Val Asp Cys Cys Thr Asp Pro Asp Gly Arg Ala
 675 680 685
 Val Asp Arg Ala Arg Ala Trp Ser Glu Met Val Gly Ile Gln Tyr Phe
 690 695 700
 Arg Leu Asn Pro Gln Leu Gly Ser Asp Ile Met Leu Asp Glu Val Asn
 705 710 715 720

Glu His Arg Glu Glu Phe Gln Lys Leu Val Gln Met Leu Leu Ser Pro
740 745 750

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 7 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

Asn Pro His Ser Gly Phe Arg
1 5

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

Xaa Ala Ser Xaa Gly Leu Asn Gln Val Asn Lys
1 5 10

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

Tyr Gly Ala Ser Pro Leu His Xaa Ala Lys
1 5 10

39

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 7 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (iii) HYPOTHETICAL: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
- Asp Asn Met Glu Met Ile Lys
1 5
- (2) INFORMATION FOR SEQ ID NO:7:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 5 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (iii) HYPOTHETICAL: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
- Gly Val Tyr Phe Arg
1 5
- (2) INFORMATION FOR SEQ ID NO:8:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 7 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (iii) HYPOTHETICAL: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
- Met Lys Asp Glu Val Phe Arg
1 5
- (2) INFORMATION FOR SEQ ID NO:9:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 7 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: peptide
 - (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Glu Phe Gly Glu His Thr Lys
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Met Leu Thr Gly Thr Leu Ser Asp Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Xaa Tyr Asp Ala Pro Glu Val Ile Arg
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Phe Asn Gln Asn Ile Asn Leu Lys Pro Pro Thr Gln Pro Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Xaa Xaa Gly Ala Ala Pro Thr Tyr Phe Arg Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Thr Val Phe Gly Ala Lys
1 5

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Xaa Trp Ser Glu Met Val Gly Ile Gln Tyr Phe Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2012 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 43..1224

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GAATTCGGG ACGGTGGGGC CTCCCCACCT GCCCCGCGAG AG ATG CAG TTC TTT	54
Met Gln Phe Phe	
1	
GGC CGC CTG GTC AAT ACC TTC AGT GGC GTC ACC AAC TTG TTC TCT AAC	102
Gly Arg Leu Val Asn Thr Phe Ser Gly Val Thr Asn Leu Phe Ser Asn	
5 10 15 20	
CCA TTC CGG GTG AAG GAG GTG GCT GTG GCC GAC TAC ACC TCG AGT GAC	150
Pro Phe Arg Val Lys Glu Val Ala Val Ala Asp Tyr Thr Ser Ser Asp	
25 30 35	
CGA GTT CGG GAG GAA GGG CAG CTG ATT CTG TTC CAG AAC ACT CCC AAC	198
Arg Val Arg Glu Gly Gln Leu Ile Leu Phe Gln Asn Thr Pro Asn	
40 45 50	
CGC ACC TGG GAC TGC GTC CTG GTC AAC CCC AGG AAC TCA CAG AGT GGA	246
Arg Thr Trp Asp Cys Val Leu Val Asn Pro Arg Asn Ser Gln Ser Gly	
55 60 65	
TTC CGA CTC TTC CAG CTG GAG TTG GAG GCT GAC GCC CTA GTG AAT TTC	294
Phe Arg Leu Phe Gln Leu Glu Leu Glu Ala Asp Ala Leu Val Asn Phe	
70 75 80	
CAT CAG TAT TCT TCC CAG CTG CTA CCC TTC TAT GAG AGC TCC CCT CAG	342
His Gln Tyr Ser Ser Gln Leu Leu Pro Phe Tyr Glu Ser Ser Pro Gln	
85 90 95 100	
GTC CTG CAC ACT GAG GTC CTG CAG CAC CTG ACC GAC CTC ATC CGT AAC	390
Val Leu His Thr Glu Val Leu Gln His Thr Asp Leu Ile Arg Asn	
105 110 115	
CAC CCC AGC TGG TCA GTG GCC CAC CTG GCT GTG GAG CTA GGG ATC CGC	438
His Pro Ser Trp Ser Val Ala His Leu Ala Val Glu Leu Gly Ile Arg	
120 125 130	
GAG TGC TTC CAT CAC AGC CGT ATC ATC AGC TGT GCC AAT TGC GCG GAG	486
Glu Cys Phe His His Ser Arg Ile Ile Ser Cys Ala Asn Cys Ala Glu	
135 140 145	
AAC GAG GAG GGC TGC ACA CCC CTG CAC CTG GCC TGC CGC AAG GGT GAT	534
Asn Glu Glu Gly Cys Thr Pro Leu His Leu Ala Cys Arg Lys Gly Asp	
150 155 160	
GGG GAG ATC CTG GTG GAG CTG GTG CAG TAC TGC CAC ACT CAG ATG GAT	582
Gly Glu Ile Leu Val Glu Leu Val Gln Tyr Cys His Thr Gln Met Asp	
165 170 175 180	
GTC ACC GAC TAC AAG GGA GAG ACC GTC TTC CAT TAT GCT GTC CAG GGT	630
Val Thr Asp Tyr Lys Gly Glu Thr Val Phe His Tyr Ala Val Gln Gly	
185 190 195	
GAC AAT TCT CAG GTG CTG CAG CTC CTT GGA AGG AAC GCA GTG GCT GGC	678
Asp Asn Ser Gln Val Leu Gln Leu Leu Gly Arg Asn Ala Val Ala Gly	
200 205 210	
CTG AAC CAG GTG AAT AAC CAA GGG CTG ACC CCG CTG CAC CTG GCC TGC	726
Leu Asn Gln Val Asn Asn Gln Gly Leu Thr Pro Leu His Leu Ala Cys	
215 220 225	

CAG CTG GGG AAG CAG GAG ATG GTC CGC GTG CTG CTG CTG TGC AAT GCT Gln Leu Gly Lys Gln Glu Met Val Arg Val Leu Leu Leu Cys Asn Ala 230 235 240	774
CGG TGC AAC ATC ATG GGC CCC AAC GGC TAC CCC ATC CAC TCG GCC ATG Arg Cys Asn Ile Met Gly Pro Asn Gly Tyr Pro Ile His Ser Ala Met 245 250 255 260	822
AAG TTC TCT CAG AAG GGG TGT GCG GAG ATG ATC ATC AGC ATG GAC AGC Lys Phe Ser Gln Lys Gly Cys Ala Glu Met Ile Ile Ser Met Asp Ser 265 270 275	870
AGC CAG ATC CAC AGC AAA GAC CCC CGT TAC GGA GCC AGC CCC CTC CAC Ser Gln Ile His Ser Lys Asp Pro Arg Tyr Gly Ala Ser Pro Leu His 280 285 290	918
TGG GCC AAG AAC GCA GAG ATG GCC CGC ATG CTG CTG AAA CGG GGC TGC Trp Ala Lys Asn Ala Glu Met Ala Arg Met Leu Leu Lys Arg Gly Cys 295 300 305	966
AAC GTG AAC AGC ACC AGC TCC GCG GGG AAC ACG GCC CTG CAC GTG GGG Asn Val Asn Ser Thr Ser Ser Ala Gly Asn Thr Ala Leu His Val Gly 310 315 320	1014
GTG ATG CGC AAC CGC TTC GAC TGT GCC ATA GTG CTG CTG ACC CAC GGG Val Met Arg Asn Arg Phe Asp Cys Ala Ile Val Leu Leu Thr His Gly 325 330 335 340	1062
GCC AAC GCG GAT GCC CGC GGA GAG CAC GGC AAC ACC CCG CTG CAC CTG Ala Asn Ala Asp Ala Arg Gly Glu His Gly Asn Thr Pro Leu His Leu 345 350 355	1110
GCC ATG TCG AAA GAC AAC GTG GAG ATG ATC AAG GCC CTC ATC GTG TTC Ala Met Ser Lys Asp Asn Val Glu Met Ile Lys Ala Leu Ile Val Phe 360 365 370	1158
GGA GCA GAA GTG GAC ACC CCG AAT GAC TTT GGG GAG ACT CCT ACA TTC Gly Ala Glu Val Asp Thr Pro Asn Asp Phe Gly Glu Thr Pro Thr Phe 375 380 385	1206
CTA GCC TCC AAA ATC GGC AGACTTGTC A CAGGAAGGC GATCTTGACT Leu Ala Ser Lys Ile Gly 390	1254
CTGCTGAGAA CCGTGGGGGC CGAATACTGC TTCCCACCCA TCCACGGGGT CCCC GCGGAG	1314
CAGGGCTCTG CAGCGCCACA TCATCCCTTC TCCCTGGAAA GAGCTCAGCC CCCACCGATC	1374
AGCCTAAACA ACCTAGGCAG TCACCCAAGC CAGGCCGGAT GGTGGGCCTG GGGTGCGGCG	1434
TCAGATGGGT AACGCCCTGG GCCTGGAGAG GCCACCGAGC CTAGCCATGC GGCATTAGCT	1494
CTAGCTCTCA CTCCCTAATC CGTCCTTCTT AGCTGCGCAC ACACCACACG CCCCCTCCCC	1554
TGCACCCTGT CCCC GGCCCTC TCTCAGCCAC TCTTCTGCTT CCCTTGTTCA CTGTGCAGCC	1614
GTGTGCCCTG GGGAGGGGGA GACACCGCTT CGCAGCCCTC GGTTCTGCTT TGCTGCTTCT	1674
AGACTCTGCA CAGTGGTGGG GGGCTGTGAG AGTTGGGGTC ACGCGGGCTG CTGCACCAGG	1734
CACCTGGGGA CTGGGCTGCT TGTCAGGAGG GGCAGCTAGT CAGTTGGGTG GACGTCGGGC	1794
AGGCCTTGGA CACAAAGGAA GACATGGACA GAGTGGATGG TGGGCCTGAT CCCGGAGGCC	1854
ACTGGGATTT CCAGACCTGG GATCAGGACG AGGGATGTCT CCTTTCATCC ATGGACTTAA	1914
ACCCCGAGGA ACGTCCTGAC TCAGCCTTTT GACTAAATGA CCTTGGGTGA ATTATGGACC	1974

CTCTTAGAGC CTCACCTGTC AATAGGGAAT AAGAATTC

2012

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 394 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Gln Phe Phe Gly Arg Leu Val Asn Thr Phe Ser Gly Val Thr Asn
 1 5 10 15
 Leu Phe Ser Asn Pro Phe Arg Val Lys Glu Val Ala Val Ala Asp Tyr
 20 25 30
 Thr Ser Ser Asp Arg Val Arg Glu Glu Gly Gln Leu Ile Leu Phe Gln
 35 40 45
 Asn Thr Pro Asn Arg Thr Trp Asp Cys Val Leu Val Asn Pro Arg Asn
 50 55 60
 Ser Gln Ser Gly Phe Arg Leu Phe Gln Leu Glu Leu Glu Ala Asp Ala
 65 70 75 80
 Leu Val Asn Phe His Gln Tyr Ser Ser Gln Leu Leu Pro Phe Tyr Glu
 85 90 95
 Ser Ser Pro Gln Val Leu His Thr Glu Val Leu Gln His Leu Thr Asp
 100 105 110
 Leu Ile Arg Asn His Pro Ser Trp Ser Val Ala His Leu Ala Val Glu
 115 120 125
 Leu Gly Ile Arg Glu Cys Phe His His Ser Arg Ile Ile Ser Cys Ala
 130 135 140
 Asn Cys Ala Glu Asn Glu Glu Gly Cys Thr Pro Leu His Leu Ala Cys
 145 150 155 160
 Arg Lys Gly Asp Gly Glu Ile Leu Val Glu Leu Val Gln Tyr Cys His
 165 170 175
 Thr Gln Met Asp Val Thr Asp Tyr Lys Gly Glu Thr Val Phe His Tyr
 180 185 190
 Ala Val Gln Gly Asp Asn Ser Gln Val Leu Gln Leu Leu Gly Arg Asn
 195 200 205
 Ala Val Ala Gly Leu Asn Gln Val Asn Asn Gln Gly Leu Thr Pro Leu
 210 215 220
 His Leu Ala Cys Gln Leu Gly Lys Gln Glu Met Val Arg Val Leu Leu
 225 230 235 240
 Leu Cys Asn Ala Arg Cys Asn Ile Met Gly Pro Asn Gly Tyr Pro Ile
 245 250 255
 His Ser Ala Met Lys Phe Ser Gln Lys Gly Cys Ala Glu Met Ile Ile
 260 265 270
 Ser Met Asp Ser Ser Gln Ile His Ser Lys Asp Pro Arg Tyr Gly Ala
 275 280 285

Ser Pro Leu His Trp Ala Lys Asn Ala Glu Met Ala Arg Met Leu Leu
 290 295 300

Lys Arg Gly Cys Asn Val Asn Ser Thr Ser Ser Ala Gly Asn Thr Ala
 305 310 315 320

Leu His Val Gly Val Met Arg Asn Arg Phe Asp Cys Ala Ile Val Leu
 325 330 335

Leu Thr His Gly Ala Asn Ala Asp Ala Arg Gly Glu His Gly Asn Thr
 340 345 350

Pro Leu His Leu Ala Met Ser Lys Asp Asn Val Glu Met Ile Lys Ala
 355 360 365

Leu Ile Val Phe Gly Ala Glu Val Asp Thr Pro Asn Asp Phe Gly Glu
 370 375 380

Thr Pro Thr Phe Leu Ala Ser Lys Ile Gly
 385 390

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1277 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 396..1271

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GAATTCTTAG GCCCAGGTG GTTATTGCAG CATCGGCTCC GATGCAAGAA GAAGCACTTT 60

GTCTGAAGAG GACACGCAAG GGTATTCATG CCTTGGGGTT TCAAGAGGAA GAGATTGAGG 120

GGAACCTGGG AGCTGGCTGG GCAGGGTGGG GAGCCCTTCC CAGAGCAGTG GGCCCCCCTT 180

TCCACTCCAG CCCATTCTC TCCTGTGGCC TGTGGCTCAG CTTTCTCCTG GGACAGAGTC 240

CTTCCTGTGG GGAAGGGACA GATGACAGGG GGAGTGGGGG GATGAGGGCG TGGCCGTGGG 300

CGAGGCACAG CCCAGGTTTG ATCTAGGGAC CTCTGGGGTA GCAGGGCTTG GGGACCCACC 360

TGACCACAGC ATGCCCTGCT CTGTGCCTCA CAGAA CTA CAG GAT CTC ATG CAC 413
 Leu Gln Asp Leu Met His
 1 5

ATC TCA CGG GCC CGG AAG CCA GCG TTC ATC CTG GGC TCC ATG AGG GAC 461
 Ile Ser Arg Ala Arg Lys Pro Ala Phe Ile Leu Gly Ser Met Arg Asp
 10 15 20

GAG AAG CGG ACC CAC GAC CAC CTG CTG TGC CTG GAT GGA GGA GGA GTG 509
 Glu Lys Arg Thr His Asp His Leu Leu Cys Leu Asp Gly Gly Gly Val
 25 30 35

AAA GGC CTC ATC ATC ATC CAG CTC CTC ATC GCC ATC GAG AAG GCC TCG Lys Gly Leu Ile Ile Ile Gln Leu Leu Ile Ala Ile Glu Lys Ala Ser 40 45 50	557
GGT GTG GCC ACC AAG GAC CTG TTT GAC TGG GTG GCG GGC ACC AGC ACT Gly Val Ala Thr Lys Asp Leu Phe Asp Trp Val Ala Gly Thr Ser Thr 55 60 65 70	605
GGA GGC ATC CTG GCC CTG GCC ATT CTG CAC AGT AAG TCC ATG GCC TAC Gly Gly Ile Leu Ala Leu Ala Ile Leu His Ser Lys Ser Met Ala Tyr 75 80 85	653
ATG CGC GGC ATG TAC TTT CGC ATG AAG GAT GAG GTG TTC CGG GGC TCC Met Arg Gly Met Tyr Phe Arg Met Lys Asp Glu Val Phe Arg Gly Ser 90 95 100	701
AGG CCC TAC GAG TCG GGG CCC CTG GAG GAG TTC CTG AAG CGG GAG TTT Arg Pro Tyr Glu Ser Gly Pro Leu Glu Glu Phe Leu Lys Arg Glu Phe 105 110 115	749
GGG GAG CAC ACC AAG ATG ACG GAC GTC AGG AAA CCC AAG GTG ATG CTG Gly Glu His Thr Lys Met Thr Asp Val Arg Lys Pro Lys Val Met Leu 120 125 130	797
ACA GGG ACA CTG TCT GAC CGG CAG CCG GCT GAA CTC CAC CTC TTC CGG Thr Gly Thr Leu Ser Asp Arg Gln Pro Ala Glu Leu His Leu Phe Arg 135 140 145 150	845
AAC TAC GAT GCT CCA GAA ACT GTC CGG GAG CCT CGT TTC AAC CAG AAC Asn Tyr Asp Ala Pro Glu Thr Val Arg Glu Pro Arg Phe Asn Gln Asn 155 160 165	893
GTT AAC CTC AGG CCT CCA GCT CAG CCC TCA GAC CAG CTG GTG TGG CGG Val Asn Leu Arg Pro Pro Ala Gln Pro Ser Asp Gln Leu Val Trp Arg 170 175 180	941
GCG GCC CGA AGC AGC GGG GCA GCT CCT ACT TAC TTC CGA CCC AAT GGG Ala Ala Arg Ser Ser Gly Ala Ala Pro Thr Tyr Phe Arg Pro Asn Gly 185 190 195	989
CGC TTC CTG GAC GGT GGG CTG TTG GCC AAC AAC CCC ACG CTG GAT GCC Arg Phe Leu Asp Gly Gly Leu Leu Ala Asn Asn Pro Thr Leu Asp Ala 200 205 210	1037
ATG ACC GAG ATC CAT GAG TAC AAT CAG GAC CTG ATC CGC AAG GGT CAG Met Thr Glu Ile His Glu Tyr Asn Gln Asp Leu Ile Arg Lys Gly Gln 215 220 225 230	1085
GCC AAC AAG GTG AAG AAA CTC TCC ATC GTT GTC TCC CTG GGG ACA GGG Ala Asn Lys Val Lys Lys Leu Ser Ile Val Val Ser Leu Gly Thr Gly 235 240 245	1133
AGG TCC CCA CAA GTG CCT GTG ACC TGT GTG GAT GTC TTC CGT CCC AGC Arg Ser Pro Gln Val Pro Val Thr Cys Val Asp Val Phe Arg Pro Ser 250 255 260	1181
AAC CCC TGG GAG CTG GCC AAG ACT GTT TTT GGG GCC AAG GAA CTG GGC Asn Pro Trp Glu Leu Ala Lys Thr Val Phe Gly Ala Lys Glu Leu Gly 265 270 275	1229
AAG ATG GTG GTG GAC TGT TGC ACG GAT CCA GAC GGG CGG CCG Lys Met Val Val Asp Cys Cys Thr Asp Pro Asp Gly Arg Pro 280 285 290	1271
GAATTC	1277

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 292 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```

Leu Gln Asp Leu Met His Ile Ser Arg Ala Arg Lys Pro Ala Phe Ile
 1             5             10             15
Leu Gly Ser Met Arg Asp Glu Lys Arg Thr His Asp His Leu Leu Cys
      20             25             30
Leu Asp Gly Gly Gly Val Lys Gly Leu Ile Ile Ile Gln Leu Leu Ile
      35             40             45
Ala Ile Glu Lys Ala Ser Gly Val Ala Thr Lys Asp Leu Phe Asp Trp
      50             55             60
Val Ala Gly Thr Ser Thr Gly Gly Ile Leu Ala Leu Ala Ile Leu His
      65             70             75             80
Ser Lys Ser Met Ala Tyr Met Arg Gly Met Tyr Phe Arg Met Lys Asp
      85             90             95
Glu Val Phe Arg Gly Ser Arg Pro Tyr Glu Ser Gly Pro Leu Glu Glu
      100            105            110
Phe Leu Lys Arg Glu Phe Gly Glu His Thr Lys Met Thr Asp Val Arg
      115            120            125
Lys Pro Lys Val Met Leu Thr Gly Thr Leu Ser Asp Arg Gln Pro Ala
      130            135            140
Glu Leu His Leu Phe Arg Asn Tyr Asp Ala Pro Glu Thr Val Arg Glu
      145            150            155            160
Pro Arg Phe Asn Gln Asn Val Asn Leu Arg Pro Pro Ala Gln Pro Ser
      165            170            175
Asp Gln Leu Val Trp Arg Ala Ala Arg Ser Ser Gly Ala Ala Pro Thr
      180            185            190
Tyr Phe Arg Pro Asn Gly Arg Phe Leu Asp Gly Gly Leu Leu Ala Asn
      195            200            205
Asn Pro Thr Leu Asp Ala Met Thr Glu Ile His Glu Tyr Asn Gln Asp
      210            215            220
Leu Ile Arg Lys Gly Gln Ala Asn Lys Val Lys Lys Leu Ser Ile Val
      225            230            235            240
Val Ser Leu Gly Thr Gly Arg Ser Pro Gln Val Pro Val Thr Cys Val
      245            250            255
Asp Val Phe Arg Pro Ser Asn Pro Trp Glu Leu Ala Lys Thr Val Phe
      260            265            270
Gly Ala Lys Glu Leu Gly Lys Met Val Val Asp Cys Cys Thr Asp Pro
      275            280            285
Asp Gly Arg Pro
      290

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(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2109 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 43..2103

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GAATTCCGGG ACGGTGGGGC CTCCCCACCT GCCCCGCAGA AG ATG CAG TTC TTT	54
Met Gln Phe Phe	
1	
GGC CGC CTG GTC AAT ACC TTC AGT GGC GTC ACC AAC TTG TTC TCT AAC	102
Gly Arg Leu Val Asn Thr Phe Ser Gly Val Thr Asn Leu Phe Ser Asn	
5 10 15 20	
CCA TTC CGG GTG AAG GAG GTG GCT GTG GCC GAC TAC ACC TCG AGT GAC	150
Pro Phe Arg Val Lys Glu Val Ala Val Ala Asp Tyr Thr Ser Ser Asp	
25 30 35	
CGA GTT CGG GAG GAA GGG CAG CTG ATT CTG TTC CAG AAC ACT CCC AAC	198
Arg Val Arg Glu Glu Gly Gln Leu Ile Leu Phe Gln Asn Thr Pro Asn	
40 45 50	
CGC ACC TGG GAC TGC GTC CTG GTC AAC CCC AGG AAC TCA CAG AGT GGA	246
Arg Thr Trp Asp Cys Val Leu Val Asn Pro Arg Asn Ser Gln Ser Gly	
55 60 65	
TTC CGA CTC TTC CAG CTG GAG TTG GAG GCT GAC GCC CTA GTG AAT TTC	294
Phe Arg Leu Phe Gln Leu Glu Leu Glu Ala Asp Ala Leu Val Asn Phe	
70 75 80	
CAT CAG TAT TCT TCC CAG CTG CTA CCC TTC TAT GAG AGC TCC CCT CAG	342
His Gln Tyr Ser Ser Gln Leu Leu Pro Phe Tyr Glu Ser Ser Pro Gln	
85 90 95 100	
GTC CTG CAC ACT GAG GTC CTG CAG CAC CTG ACC GAC CTC ATC CGT AAC	390
Val Leu His Thr Glu Val Leu Gln His Leu Thr Asp Leu Ile Arg Asn	
105 110 115	
CAC CCC AGC TGG TCA GTG GCC CAC CTG GCT GTG GAG CTA GGG ATC CGC	438
His Pro Ser Trp Ser Val Ala His Leu Ala Val Glu Leu Gly Ile Arg	
120 125 130	
GAG TGC TTC CAT CAC AGC CGT ATC ATC AGC TGT GCC AAT TGC GCG GAG	486
Glu Cys Phe His His Ser Arg Ile Ile Ser Cys Ala Asn Cys Ala Glu	
135 140 145	
AAC GAG GAG GGC TGC ACA CCC CTG CAC CTG GCC TGC CGC AAG GGT GAT	534
Asn Glu Glu Gly Cys Thr Pro Leu His Leu Ala Cys Arg Lys Gly Asp	
150 155 160	
GGG GAG ATC CTG GTG GAG CTG GTG CAG TAC TGC CAC ACT CAG ATG GAT	582
Gly Glu Ile Leu Val Glu Leu Val Gln Tyr Cys His Thr Gln Met Asp	
165 170 175 180	

GTC ACC GAC TAC AAG GGA GAG ACC GTC TTC CAT TAT GCT GTC CAG GGT Val Thr Asp Tyr Lys Gly Glu Thr Val Phe His Tyr Ala Val Gln Gly 185 190 195	630
GAC AAT TCT CAG GTG CTG CAG CTC CTT GGA AGG AAC GCA GTG GCT GGC Asp Asn Ser Gln Val Leu Gln Leu Leu Gly Arg Asn Ala Val Ala Gly 200 205 210	678
CTG AAC CAG GTG AAT AAC CAA GGG CTG ACC CCG CTG CAC CTG GCC TGC Leu Asn Gln Val Asn Asn Gln Gly Leu Thr Pro Leu His Leu Ala Cys 215 220 225	726
CAG CTG GGG AAG CAG GAG ATG GTC CGC GTG CTG CTG CTG TGC AAT GCT Gln Leu Gly Lys Gln Glu Met Val Arg Val Leu Leu Leu Cys Asn Ala 230 235 240	774
CGG TGC AAC ATC ATG GGC CCC AAC GGC TAC CCC ATC CAC TCG GCC ATG Arg Cys Asn Ile Met Gly Pro Asn Gly Tyr Pro Ile His Ser Ala Met 245 250 255 260	822
AAG TTC TCT CAG AAG GGG TGT GCG GAG ATG ATC ATC AGC ATG GAC AGC Lys Phe Ser Gln Lys Gly Cys Ala Glu Met Ile Ile Ser Met Asp Ser 265 270 275	870
AGC CAG ATC CAC AGC AAA GAC CCC CGT TAC GGA GCC AGC CCC CTC CAC Ser Gln Ile His Ser Lys Asp Pro Arg Tyr Gly Ala Ser Pro Leu His 280 285 290	918
TGG GCC AAG AAC GCA GAG ATG GCC CGC ATG CTG CTG AAA CGG GGC TGC Trp Ala Lys Asn Ala Glu Met Ala Arg Met Leu Leu Lys Arg Gly Cys 295 300 305	966
AAC GTG AAC AGC ACC AGC TCC GCG GGG AAC ACG GCC CTG CAC GTG GGG Asn Val Asn Ser Thr Ser Ser Ala Gly Asn Thr Ala Leu His Val Gly 310 315 320	1014
GTG ATG CGC AAC CGC TTC GAC TGT GCC ATA GTG CTG CTG ACC CAC GGG Val Met Arg Asn Arg Phe Asp Cys Ala Ile Val Leu Leu Thr His Gly 325 330 335 340	1062
GCC AAC GCG GAT GCC CGC GGA GAG CAC GGC AAC ACC CCG CTG CAC CTG Ala Asn Ala Asp Ala Arg Gly Glu His Gly Asn Thr Pro Leu His Leu 345 350 355	1110
GCC ATG TCG AAA GAC AAC GTG GAG ATG ATC AAG GCC CTC ATC GTG TTC Ala Met Ser Lys Asp Asn Val Glu Met Ile Lys Ala Leu Ile Val Phe 360 365 370	1158
GGA GCA GAA GTG GAC ACC CCG AAT GAC TTT GGG GAG ACT CCT ACA TTC Gly Ala Glu Val Asp Thr Pro Asn Asp Phe Gly Glu Thr Pro Thr Phe 375 380 385	1206
CTA GCC TCC AAA ATC GGC AAA CTA CAG GAT CTC ATG CAC ATC TCA CGG Leu Ala Ser Lys Ile Gly Lys Leu Gln Asp Leu Met His Ile Ser Arg 390 395 400	1254
GCC CGG AAG CCA GCG TTC ATC CTG GGC TCC ATG AGG GAC GAG AAG CGG Ala Arg Lys Pro Ala Phe Ile Leu Gly Ser Met Arg Asp Glu Lys Arg 405 410 415 420	1302
ACC CAC GAC CAC CTG CTG TGC CTG GAT GGA GGA GGA GTG AAA GGC CTC Thr His Asp His Leu Leu Cys Leu Asp Gly Gly Gly Val Lys Gly Leu 425 430 435	1350
ATC ATC ATC CAG CTC CTC ATC GCC ATC GAG AAG GCC TCG GGT GTG GCC Ile Ile Ile Gln Leu Leu Ile Ala Ile Glu Lys Ala Ser Gly Val Ala 440 445 450	1398

ACC	AAG	GAC	CTG	TTT	GAC	TGG	GTG	GCG	GGC	ACC	AGC	ACT	GGA	GGC	ATC	1446
Thr	Lys	Asp	Leu	Phe	Asp	Trp	Val	Ala	Gly	Thr	Ser	Thr	Gly	Gly	Ile	
		455					460					465				
CTG	GCC	CTG	GCC	ATT	CTG	CAC	AGT	AAG	TCC	ATG	GCC	TAC	ATG	CGC	GGC	1494
Leu	Ala	Leu	Ala	Ile	Leu	His	Ser	Lys	Ser	Met	Ala	Tyr	Met	Arg	Gly	
	470					475					480					
ATG	TAC	TTT	CGC	ATG	AAG	GAT	GAG	GTG	TTC	CGG	GGC	TCC	AGG	CCC	TAC	1542
Met	Tyr	Phe	Arg	Met	Lys	Asp	Glu	Val	Phe	Arg	Gly	Ser	Arg	Pro	Tyr	
485					490					495					500	
GAG	TCG	GGG	CCC	CTG	GAG	GAG	TTC	CTG	AAG	CGG	GAG	TTT	GGG	GAG	CAC	1590
Glu	Ser	Gly	Pro	Leu	Glu	Glu	Phe	Leu	Lys	Arg	Glu	Phe	Gly	Glu	His	
				505					510					515		
ACC	AAG	ATG	ACG	GAC	GTC	AGG	AAA	CCC	AAG	GTG	ATG	CTG	ACA	GGG	ACA	1638
Thr	Lys	Met	Thr	Asp	Val	Arg	Lys	Pro	Lys	Val	Met	Leu	Thr	Gly	Thr	
			520					525					530			
CTG	TCT	GAC	CGG	CAG	CCG	GCT	GAA	CTC	CAC	CTC	TTC	CGG	AAC	TAC	GAT	1686
Leu	Ser	Asp	Arg	Gln	Pro	Ala	Glu	Leu	His	Leu	Phe	Arg	Asn	Tyr	Asp	
		535					540					545				
GCT	CCA	GAA	ACT	GTC	CGG	GAG	CCT	CGT	TTC	AAC	CAG	AAC	GTT	AAC	CTC	1734
Ala	Pro	Glu	Thr	Val	Arg	Glu	Pro	Arg	Phe	Asn	Gln	Asn	Val	Asn	Leu	
	550					555					560					
AGG	CCT	CCA	GCT	CAG	CCC	TCA	GAC	CAG	CTG	GTG	TGG	CGG	GCG	GCC	CGA	1782
Arg	Pro	Pro	Ala	Gln	Pro	TCA	Ser	Asp	Gln	Leu	Val	Trp	Arg	Ala	Arg	
565					570					575					580	
AGC	AGC	GGG	GCA	GCT	CCT	ACT	TAC	TTC	CGA	CCC	AAT	GGG	CGC	TTC	CTG	1830
Ser	Ser	Gly	Ala	Ala	Pro	Thr	Tyr	Phe	Arg	Pro	Asn	Gly	Arg	Phe	Leu	
				585					590					595		
GAC	GGT	GGG	CTG	TTG	GCC	AAC	AAC	CCC	ACG	CTG	GAT	GCC	ATG	ACC	GAG	1878
Asp	Gly	Gly	Leu	Leu	Ala	Asn	Asn	Pro	Thr	Leu	Asp	Ala	Met	Thr	Glu	
			600					605					610			
ATC	CAT	GAG	TAC	AAT	CAG	GAC	CTG	ATC	CGC	AAG	GGT	CAG	GCC	AAC	AAG	1926
Ile	His	Glu	Tyr	Asn	Gln	Asp	Leu	Ile	Arg	Lys	Gly	Gln	Ala	Asn	Lys	
		615					620					625				
GTG	AAG	AAA	CTC	TCC	ATC	GTT	GTG	TCC	CTG	GGG	ACA	GGG	AGG	TCC	CCA	1974
Val	Lys	Lys	Leu	Ser	Ile	Val	Val	Ser	Leu	Gly	Thr	Gly	Arg	Ser	Pro	
	630					635					640					
CAA	GTG	CCT	GTG	ACC	TGT	GTG	GAT	GTC	TTC	CGT	CCC	AGC	AAC	CCC	TGG	2022
Gln	Val	Pro	Val	Thr	Cys	Val	Asp	Val	Phe	Arg	Pro	Ser	Asn	Pro	Trp	
645					650					655					660	
GAG	CTG	GCC	AAG	ACT	GTT	TTT	GGG	GCC	AAG	GAA	CTG	GGC	AAG	ATG	GTG	2070
Glu	Leu	Ala	Lys	Thr	Val	Phe	Gly	Ala	Lys	Glu	Leu	Gly	Lys	Met	Val	
				665					670					675		
GTG	GAC	TGT	TGC	ACG	GAT	CCA	GAC	GGG	CGG	CCG	GAATTC					2109
Val	Asp	Cys	Cys	Thr	Asp	Pro	Asp	Gly	Arg	Pro						
			680					685								

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 687 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

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Met Gln Phe Phe Gly Arg Leu Val Asn Thr Phe Ser Gly Val Thr Asn
 1           5           10           15
Leu Phe Ser Asn Pro Phe Arg Val Lys Glu Val Ala Val Ala Asp Tyr
          20           25           30
Thr Ser Ser Asp Arg Val Arg Glu Glu Gly Gln Leu Ile Leu Phe Gln
          35           40           45
Asn Thr Pro Asn Arg Thr Trp Asp Cys Val Leu Val Asn Pro Arg Asn
          50           55           60
Ser Gln Ser Gly Phe Arg Leu Phe Gln Leu Glu Leu Glu Ala Asp Ala
          65           70           75           80
Leu Val Asn Phe His Gln Tyr Ser Ser Gln Leu Leu Pro Phe Tyr Glu
          85           90           95
Ser Ser Pro Gln Val Leu His Thr Glu Val Leu Gln His Leu Thr Asp
          100          105          110
Leu Ile Arg Asn His Pro Ser Trp Ser Val Ala His Leu Ala Val Glu
          115          120          125
Leu Gly Ile Arg Glu Cys Phe His His Ser Arg Ile Ile Ser Cys Ala
          130          135          140
Asn Cys Ala Glu Asn Glu Glu Gly Cys Thr Pro Leu His Leu Ala Cys
          145          150          155          160
Arg Lys Gly Asp Gly Glu Ile Leu Val Glu Leu Val Gln Tyr Cys His
          165          170          175
Thr Gln Met Asp Val Thr Asp Tyr Lys Gly Glu Thr Val Phe His Tyr
          180          185          190
Ala Val Gln Gly Asp Asn Ser Gln Val Leu Gln Leu Leu Gly Arg Asn
          195          200          205
Ala Val Ala Gly Leu Asn Gln Val Asn Asn Gln Gly Leu Thr Pro Leu
          210          215          220
His Leu Ala Cys Gln Leu Gly Lys Gln Glu Met Val Arg Val Leu Leu
          225          230          235          240
Leu Cys Asn Ala Arg Cys Asn Ile Met Gly Pro Asn Gly Tyr Pro Ile
          245          250          255
His Ser Ala Met Lys Phe Ser Gln Lys Gly Cys Ala Glu Met Ile Ile
          260          265          270
Ser Met Asp Ser Ser Gln Ile His Ser Lys Asp Pro Arg Tyr Gly Ala
          275          280          285
Ser Pro Leu His Trp Ala Lys Asn Ala Glu Met Ala Arg Met Leu Leu
          290          295          300
Lys Arg Gly Cys Asn Val Asn Ser Thr Ser Ser Ala Gly Asn Thr Ala
          305          310          315          320
Leu His Val Gly Val Met Arg Asn Arg Phe Asp Cys Ala Ile Val Leu
          325          330          335

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Leu Thr His Gly Ala Asn Ala Asp Ala Arg Gly Glu His Gly Asn Thr
 340 345 350
 Pro Leu His Leu Ala Met Ser Lys Asp Asn Val Glu Met Ile Lys Ala
 355 360 365
 Leu Ile Val Phe Gly Ala Glu Val Asp Thr Pro Asn Asp Phe Gly Glu
 370 375 380
 Thr Pro Thr Phe Leu Ala Ser Lys Ile Gly Lys Leu Gln Asp Leu Met
 385 390 395 400
 His Ile Ser Arg Ala Arg Lys Pro Ala Phe Ile Leu Gly Ser Met Arg
 405 410 415
 Asp Glu Lys Arg Thr His Asp His Leu Leu Cys Leu Asp Gly Gly Gly
 420 425 430
 Val Lys Gly Leu Ile Ile Ile Gln Leu Leu Ile Ala Ile Glu Lys Ala
 435 440 445
 Ser Gly Val Ala Thr Lys Asp Leu Phe Asp Trp Val Ala Gly Thr Ser
 450 455 460
 Thr Gly Gly Ile Leu Ala Leu Ala Ile Leu His Ser Lys Ser Met Ala
 465 470 475 480
 Tyr Met Arg Gly Met Tyr Phe Arg Met Lys Asp Glu Val Phe Arg Gly
 485 490 495
 Ser Arg Pro Tyr Glu Ser Gly Pro Leu Glu Glu Phe Leu Lys Arg Glu
 500 505 510
 Phe Gly Glu His Thr Lys Met Thr Asp Val Arg Lys Pro Lys Val Met
 515 520 525
 Leu Thr Gly Thr Leu Ser Asp Arg Gln Pro Ala Glu Leu His Leu Phe
 530 535 540
 Arg Asn Tyr Asp Ala Pro Glu Thr Val Arg Glu Pro Arg Phe Asn Gln
 545 550 555 560
 Asn Val Asn Leu Arg Pro Pro Ala Gln Pro Ser Asp Gln Leu Val Trp
 565 570 575
 Arg Ala Ala Arg Ser Ser Gly Ala Ala Pro Thr Tyr Phe Arg Pro Asn
 580 585 590
 Gly Arg Phe Leu Asp Gly Gly Leu Leu Ala Asn Asn Pro Thr Leu Asp
 595 600 605
 Ala Met Thr Glu Ile His Glu Tyr Asn Gln Asp Leu Ile Arg Lys Gly
 610 615 620
 Gln Ala Asn Lys Val Lys Lys Leu Ser Ile Val Val Ser Leu Gly Thr
 625 630 635 640
 Gly Arg Ser Pro Gln Val Pro Val Thr Cys Val Asp Val Phe Arg Pro
 645 650 655
 Ser Asn Pro Trp Glu Leu Ala Lys Thr Val Phe Gly Ala Lys Glu Leu
 660 665 670
 Gly Lys Met Val Val Asp Cys Cys Thr Asp Pro Asp Gly Arg Pro
 675 680 685

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 2112 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 43..2106
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GAATTCCGGG ACGGTGGGGC CTCCCCACCT GCCCCGCAGA AG ATG CAG TTC TTT	54
Met Gln Phe Phe	
1	
GGC CGC CTG GTC AAT ACC TTC AGT GGC GTC ACC AAC TTG TTC TCT AAC	102
Gly Arg Leu Val Asn Thr Phe Ser Gly Val Thr Asn Leu Phe Ser Asn	
5 10 15 20	
CCA TTC CGG GTG AAG GAG GTG GCT GTG GCC GAC TAC ACC TCG AGT GAC	150
Pro Phe Arg Val Lys Glu Val Ala Val Ala Asp Tyr Thr Ser Ser Asp	
25 30 35	
CGA GTT CGG GAG GAA GGG CAG CTG ATT CTG TTC CAG AAC ACT CCC AAC	198
Arg Val Arg Glu Glu Gly Gln Leu Ile Leu Phe Gln Asn Thr Pro Asn	
40 45 50	
CGC ACC TGG GAC TGC GTC CTG GTC AAC CCC AGG AAC TCA CAG AGT GGA	246
Arg Thr Trp Asp Cys Val Leu Val Asn Pro Arg Asn Ser Gln Ser Gly	
55 60 65	
TTC CGA CTC TTC CAG CTG GAG TTG GAG GCT GAC GCC CTA GTG AAT TTC	294
Phe Arg Leu Phe Gln Leu Glu Leu Glu Ala Asp Ala Leu Val Asn Phe	
70 75 80	
CAT CAG TAT TCT TCC CAG CTG CTA CCC TTC TAT GAG AGC TCC CCT CAG	342
His Gln Tyr Ser Ser Gln Leu Leu Pro Phe Tyr Glu Ser Ser Pro Gln	
85 90 95 100	
GTC CTG CAC ACT GAG GTC CTG CAG CAC CTG ACC GAC CTC ATC CGT AAC	390
Val Leu His Thr Glu Val Leu Gln His Leu Thr Asp Leu Ile Arg Asn	
105 110 115	
CAC CCC AGC TGG TCA GTG GCC CAC CTG GCT GTG GAG CTA GGG ATC CGC	438
His Pro Ser Trp Ser Val Ala His Leu Ala Val Glu Leu Gly Ile Arg	
120 125 130	
GAG TGC TTC CAT CAC AGC CGT ATC ATC AGC TGT GCC AAT TGC GCG GAG	486
Glu Cys Phe His His Ser Arg Ile Ile Ser Cys Ala Asn Cys Ala Glu	
135 140 145	
AAC GAG GAG GGC TGC ACA CCC CTG CAC CTG GCC TGC CGC AAG GGT GAT	534
Asn Glu Glu Gly Cys Thr Pro Leu His Leu Ala Cys Arg Lys Gly Asp	
150 155 160	
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165 170 175 180	

GTC ACC GAC TAC AAG GGA GAG ACC GTC TTC CAT TAT GCT GTC CAG GGT Val Thr Asp Tyr Lys Gly Glu Thr Val Phe His Tyr Ala Val Gln Gly 185 190 195	630
GAC AAT TCT CAG GTG CTG CAG CTC CTT GGA AGG AAC GCA GTG GCT GGC Asp Asn Ser Gln Val Leu Gln Leu Leu Gly Arg Asn Ala Val Ala Gly 200 205 210	678
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CGG TGC AAC ATC ATG GGC CCC AAC GGC TAC CCC ATC CAC TCG GCC ATG Arg Cys Asn Ile Met Gly Pro Asn Gly Tyr Pro Ile His Ser Ala Met 245 250 255 260	822
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TGG GCC AAG AAC GCA GAG ATG GCC CGC ATG CTG CTG AAA CGG GGC TGC Trp Ala Lys Asn Ala Glu Met Ala Arg Met Leu Leu Lys Arg Gly Cys 295 300 305	966
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GCC AAC GCG GAT GCC CGC GGA GAG CAC GGC AAC ACC CCG CTG CAC CTG Ala Asn Ala Asp Ala Arg Gly Glu His Gly Asn Thr Pro Leu His Leu 345 350 355	1110
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CGG ACC CAC GAC CAC CTG CTG TGC CTG GAT GGA GGA GGA GTG AAA GGC Arg Thr His Asp His Leu Leu Cys Leu Asp Gly Gly Gly Val Lys Gly 425 430 435	1350
CTC ATC ATC ATC CAG CTC CTC ATC GCC ATC GAG AAG GCC TCG GGT GTG Leu Ile Ile Ile Gln Leu Leu Ile Ala Ile Glu Lys Ala Ser Gly Val 440 445 450	1398

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CCA CAA GTG CCT GTG ACC TGT GTG GAT GTC TTC CGT CCC AGC AAC CCC Pro Gln Val Pro Val Thr Cys Val Asp Val Phe Arg Pro Ser Asn Pro 645 650 655 660	2022
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(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 688 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

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Met Gln Phe Phe Gly Arg Leu Val Asn Thr Phe Ser Gly Val Thr Asn
 1           5           10           15
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           20           25           30
Thr Ser Ser Asp Arg Val Arg Glu Glu Gly Gln Leu Ile Leu Phe Gln
           35           40           45
Asn Thr Pro Asn Arg Thr Trp Asp Cys Val Leu Val Asn Pro Arg Asn
 50           55           60
Ser Gln Ser Gly Phe Arg Leu Phe Gln Leu Glu Leu Glu Ala Asp Ala
 65           70           75           80
Leu Val Asn Phe His Gln Tyr Ser Ser Gln Leu Leu Pro Phe Tyr Glu
           85           90           95
Ser Ser Pro Gln Val Leu His Thr Glu Val Leu Gln His Leu Thr Asp
           100          105          110
Leu Ile Arg Asn His Pro Ser Trp Ser Val Ala His Leu Ala Val Glu
          115          120          125
Leu Gly Ile Arg Glu Cys Phe His His Ser Arg Ile Ile Ser Cys Ala
          130          135          140
Asn Cys Ala Glu Asn Glu Glu Gly Cys Thr Pro Leu His Leu Ala Cys
          145          150          155          160
Arg Lys Gly Asp Gly Glu Ile Leu Val Glu Leu Val Gln Tyr Cys His
          165          170          175
Thr Gln Met Asp Val Thr Asp Tyr Lys Gly Glu Thr Val Phe His Tyr
          180          185          190
Ala Val Gln Gly Asp Asn Ser Gln Val Leu Gln Leu Leu Gly Arg Asn
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Ala Val Ala Gly Leu Asn Gln Val Asn Asn Gln Gly Leu Thr Pro Leu
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Leu Cys Asn Ala Arg Cys Asn Ile Met Gly Pro Asn Gly Tyr Pro Ile
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His Ser Ala Met Lys Phe Ser Gln Lys Gly Cys Ala Glu Met Ile Ile
          260          265          270
Ser Met Asp Ser Ser Gln Ile His Ser Lys Asp Pro Arg Tyr Gly Ala
          275          280          285
Ser Pro Leu His Trp Ala Lys Asn Ala Glu Met Ala Arg Met Leu Leu
          290          295          300
Lys Arg Gly Cys Asn Val Asn Ser Thr Ser Ser Ala Gly Asn Thr Ala
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Leu His Val Gly Val Met Arg Asn Arg Phe Asp Cys Ala Ile Val Leu
          325          330          335

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 Pro Leu His Leu Ala Met Ser Lys Asp Asn Val Glu Met Ile Lys Ala
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 Leu Ile Val Phe Gly Ala Glu Val Asp Thr Pro Asn Asp Phe Gly Glu
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 405 410 415
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 420 425 430
 Gly Val Lys Gly Leu Ile Ile Ile Gln Leu Leu Ile Ala Ile Glu Lys
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 Ser Thr Gly Gly Ile Leu Ala Leu Ala Ile Leu His Ser Lys Ser Met
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 485 490 495
 Gly Ser Arg Pro Tyr Glu Ser Gly Pro Leu Glu Glu Phe Leu Lys Arg
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 610 615 620
 Gly Gln Ala Asn Lys Val Lys Lys Leu Ser Ile Val Val Ser Leu Gly
 625 630 635 640
 Thr Gly Arg Ser Pro Gln Val Pro Val Thr Cys Val Asp Val Phe Arg
 645 650 655
 Pro Ser Asn Pro Trp Glu Leu Ala Lys Thr Val Phe Gly Ala Lys Glu
 660 665 670
 Leu Gly Lys Met Val Val Asp Cys Cys Thr Asp Pro Asp Gly Arg Pro
 675 680 685

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotides

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CATGGGACCC GCTGGCTTTC C

21

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotides

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GGCAGGAACC GCCACTGGGG GC

22

WHAT IS CLAIMED IS:

1. A composition comprising a purified phospholipase enzyme characterized by (a) activity in the absence of calcium; (b) a molecular weight of 86 kD on SDS-PAGE; and (c) the presence of one or more amino acid sequences selected from the group consisting of YGASPLHXAK, MKDEVFR, EFGEHTK, VMLTGTLSDR, XXGAAPTYFRP and TVFGAK, wherein X represents any amino acid residue.
2. The composition of claim 1 wherein said enzyme is further characterized by activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine.
3. The composition of claim 2 wherein said enzyme has a specific activity of about 1 μ mol to about 20 μ mol per minute per milligram.
4. The composition of claim 1 wherein said enzyme is further characterized by a pH optimum of 6.
5. The composition of claim 1 wherein said enzyme is further characterized by the absence of stimulation by adenosine triphosphate.
6. An isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of:
 - (a) the nucleotide sequence of SEQ ID NO:16;
 - (b) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:17;

- (c) a nucleotide sequence encoding a fragment of the amino acid sequence of SEQ ID NO:17 having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine;
- (d) the nucleotide sequence of SEQ ID NO:18;
- (e) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:19;
- (f) a nucleotide sequence encoding a fragment of the amino acid sequence of SEQ ID NO:19 having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine;
- (g) the nucleotide sequence of SEQ ID NO:20;
- (h) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:21;
- (i) a nucleotide sequence encoding a fragment of the amino acid sequence of SEQ ID NO:21 having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine;
- (j) the nucleotide sequence of SEQ ID NO:22;
- (k) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:23;
- (l) a nucleotide sequence encoding a fragment of the amino acid sequence of SEQ ID NO:23 having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine;
- (m) a nucleotide sequence capable of hybridizing with the sequence of any of (a)-(l) which encodes a peptide having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine; and
- (n) allelic variants of the sequence of (a), (d), (g) or (j).

7. An expression vector comprising the polynucleotide of claim 6 and an expression control sequence.
8. A host cell transformed with the vector of claim 7.
9. A process for producing a phospholipase enzyme, said process comprising:
 - (a) establishing a culture of the host cell of claim 8 in a suitable culture medium;and
 - (b) isolating said enzyme from said culture.
10. A composition comprising a peptide made according to the process of claim 9.
11. A composition comprising a peptide encoded by the polynucleotide of claim 6.
12. A composition comprising a peptide comprising an amino acid sequence selected from the group consisting of:
 - (a) the amino acid sequence of SEQ ID NO:17;
 - (b) a fragment of the amino acid sequence of SEQ ID NO:17 having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine;
 - (c) the amino acid sequence of SEQ ID NO:19;
 - (d) a fragment of the amino acid sequence of SEQ ID NO:19 having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine;
 - (e) the amino acid sequence of SEQ ID NO:21;

(f) a fragment of the amino acid sequence of SEQ ID NO:21 having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine;

(g) the amino acid sequence of SEQ ID NO:23; and

(h) a fragment of the amino acid sequence of SEQ ID NO:23 having activity in a mixed micelle assay with 1-palmitoyl-2-[¹⁴C]-arachidonyl-phosphatidylcholine.

13. A method for identifying an inhibitor of phospholipase activity, said method comprising:

(a) combining a phospholipid, a candidate inhibitor compound, and a composition comprising a phospholipase enzyme peptide; and

(b) observing whether said phospholipase enzyme peptide cleaves said phospholipid and releases fatty acid thereby,

wherein said composition is the composition of claim 1.

14. An inhibitor of phospholipase activity identified according to the method of claim 13.

15. A pharmaceutical composition comprising a therapeutically effective amount of the inhibitor of claim 14 and a pharmaceutically acceptable carrier.

16. A method of reducing inflammation comprising administering a pharmaceutical composition of claim 15 to a mammalian subject.

17. A composition comprising an antibody which binds to the peptide of the composition of claim 1.
18. The composition of claim 17 wherein said antibody is polyclonal.
19. The composition of claim 17 wherein said antibody is monoclonal.
20. The polynucleotide of claim 6 comprising the nucleotide sequence of SEQ ID NO:16.
21. The polynucleotide of claim 6 comprising a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:17.
22. The polynucleotide of claim 6 comprising the nucleotide sequence of SEQ ID NO:18.
23. The polynucleotide of claim 6 comprising a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:19.
24. The polynucleotide of claim 6 comprising the nucleotide sequence of SEQ ID NO:20.
25. The polynucleotide of claim 6 comprising a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:21.

26. The polynucleotide of claim 6 comprising the nucleotide sequence of SEQ ID NO:22.
27. The polynucleotide of claim 6 comprising a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:23.
28. A composition comprising a purified mammalian calcium independent phospholipase A_2/B enzyme.

Fig. 1

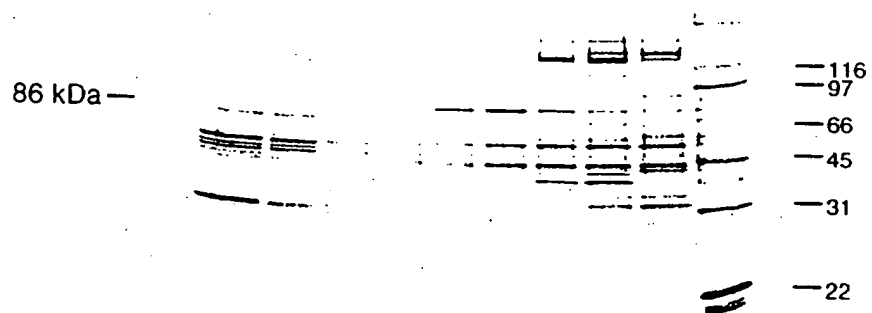
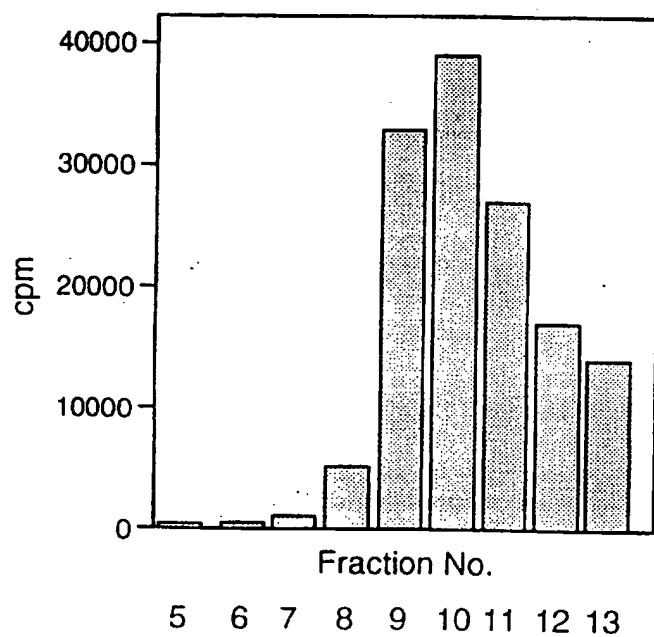


Fig. 2

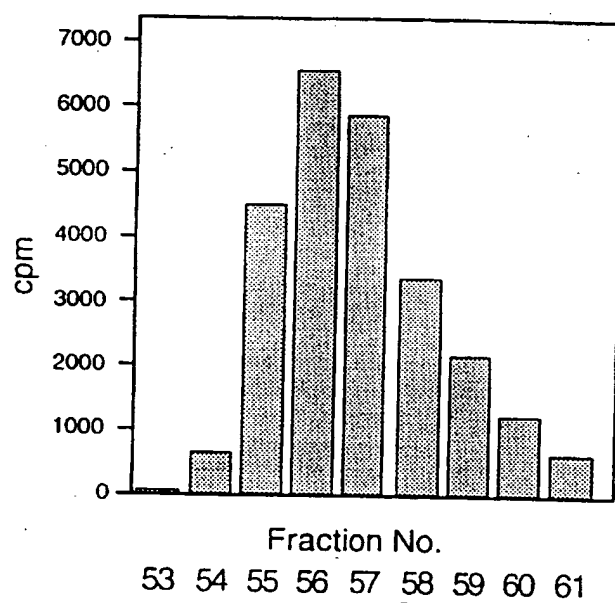
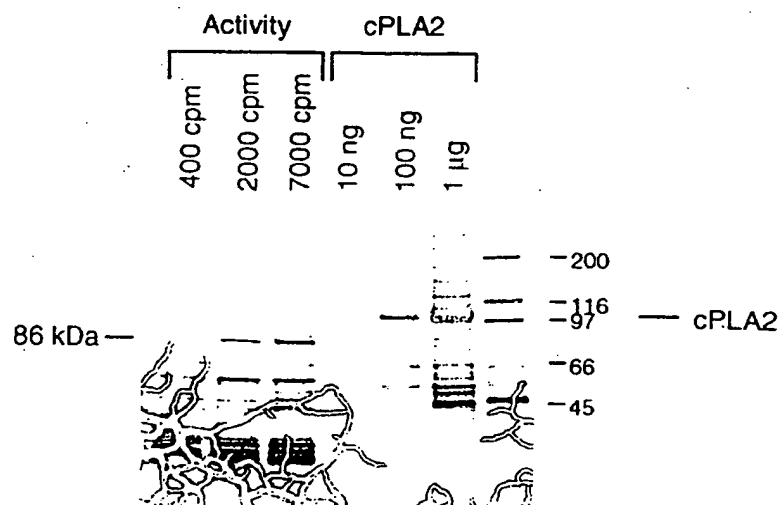


Fig. 3

3-1



3-2

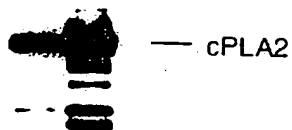


Fig. 4

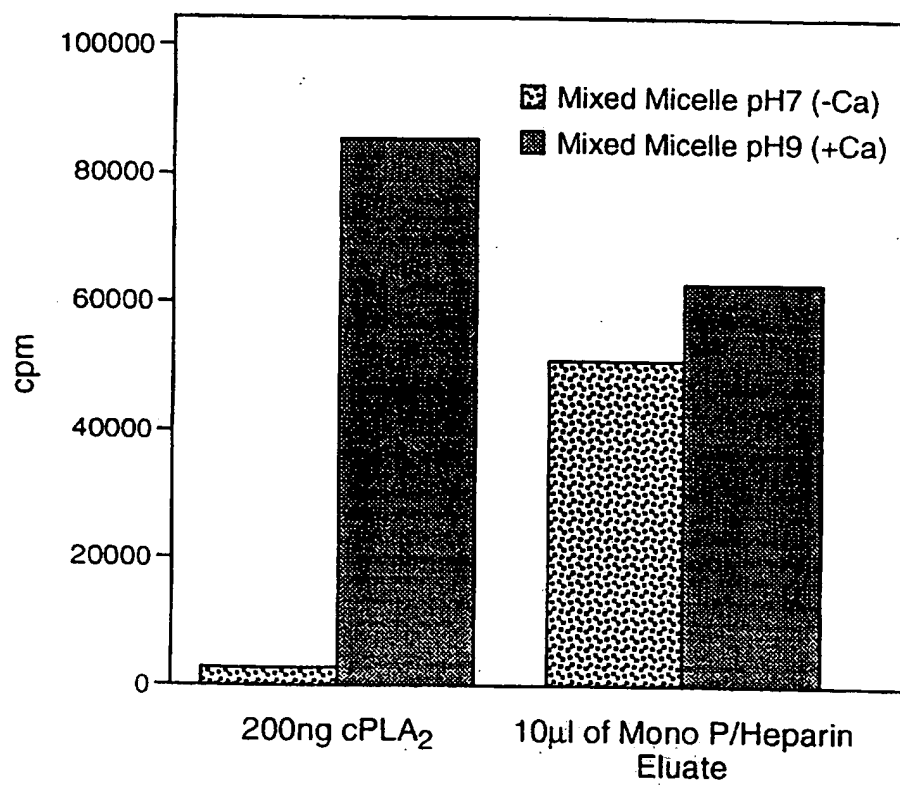


Fig. 5

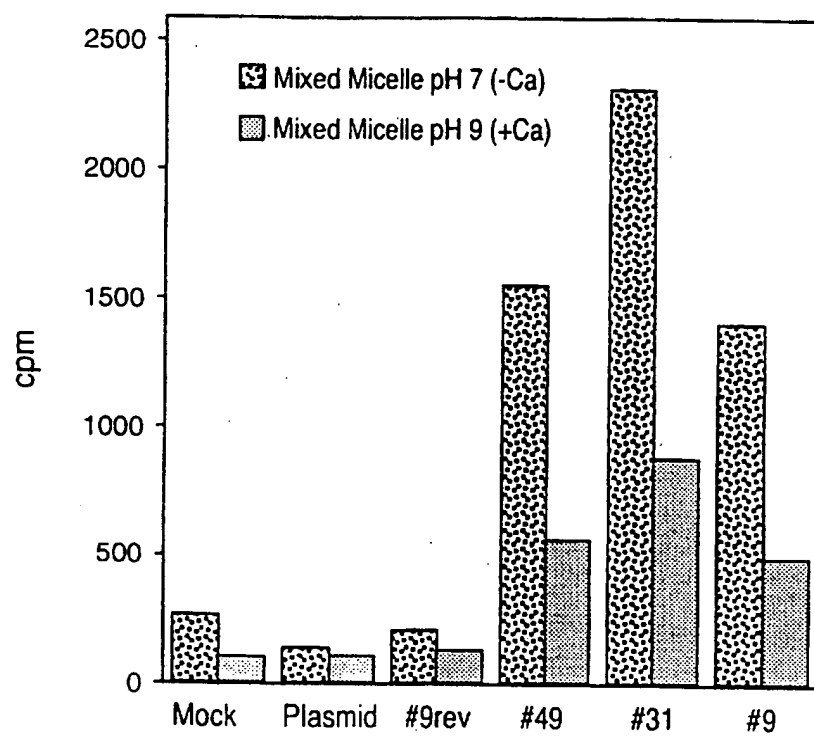


Fig. 6

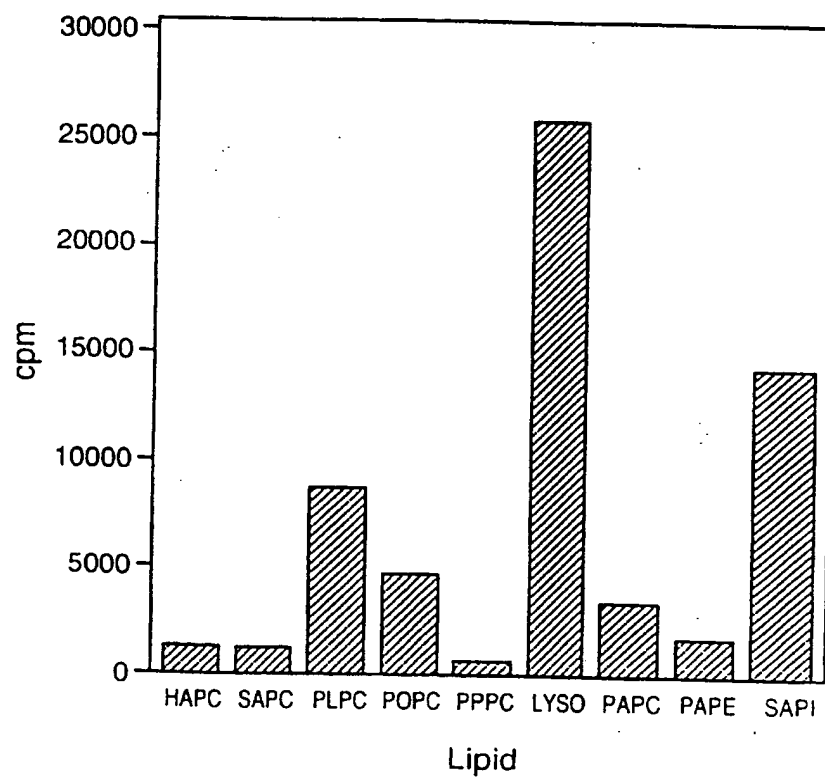
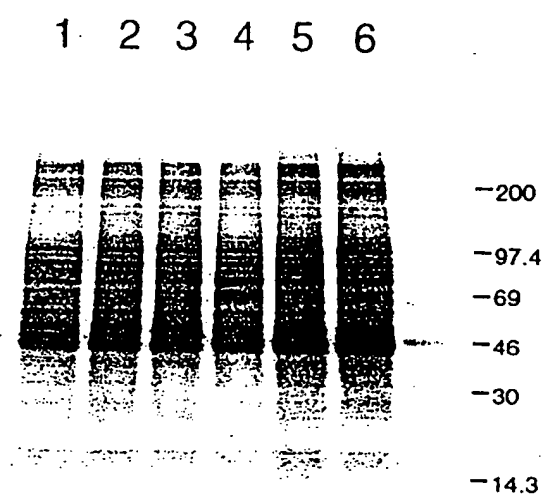


Fig. 7



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

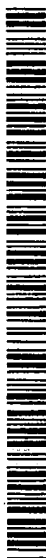


(43) International Publication Date
15 May 1997 (15.05.1997)

PCT

(10) International Publication Number
WO 97/17448 A3

- (51) International Patent Classification*: C12N 15/55, 9/16, 5/10, C07K 16/40, A61K 38/46, 39/395
- (74) Agent: BROWN, Scott, A.: Genetics Institute, Inc., 87 CambridgePark Drive, Cambridge, MA 02140 (US).
- (21) International Application Number: PCT/US96/17794
- (81) Designated States (*national*): AU, CA, JP, MX.
- (22) International Filing Date:
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- (25) Filing Language: English
- Published:
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- (88) Date of publication of the international search report:
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Previous Correction:
see PCT Gazette No. 32/1997 of 24 July 1997, Section II
- (71) Applicant: GENETICS INSTITUTE, INC. [US/US]; 87 CambridgePark Drive, Cambridge, MA 02140 (US).
- (72) Inventors: JONES, Simon; 26 Berkeley Street, Somerville, MA 02143 (US). TANG, Jin; 308 Pleasant Street, Canton, MA 02021 (US).
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



WO 97/17448 A3

(54) Title: CALCIUM INDEPENDENT CYTOSOLIC PHOSPHOLIPASE A₂/B ENZYMES

(57) Abstract: The invention provides a novel calcium-independent cytosolic phospholipase A₂/B enzyme, polynucleotides encoding such enzyme and methods for screening unknown compounds for anti-inflammatory activity mediated by the arachidonic acid cascade.

INTERNATIONAL SEARCH REPORT

International Application No

PC./US 96/17794

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/55 C12N9/16 C12N5/10 C07K16/40 A61K38/46
 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL SEQUENCE DATA LIBRARY, 2 July 1995, HEIDELBERG, GERMANY, XP002030401 HILLIER, L., ET AL.: "THE WashU-MERCK EST PROJECT" ACCESSION No.H10676 see the whole document ---	6
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 19, 5 July 1992, pages 13418-13424, XP002030402 GASSAMA-DIAGNE, A., ET AL.: "SUBSTRATE SPECIFICITY OF PHOSPHOLIPASE B FROM GUINEA PIG INTESTINE" see the whole document --- -/--	13,14,28

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- "O" document referring to an oral disclosure, use, exhibition or other means
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

29 April 1997

Date of mailing of the international search report

16. 05. 97

Name and mailing address of the ISA

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 Fax (+31-70) 340-3016

Authorized officer

Holtorf, S

INTERNATIONAL SEARCH REPORT

International Application No

PLI/US 96/17794

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 264, no. 16, 5 June 1989, pages 9470-9475, XP002030403 GASSAMA-DIAGNE, A., ET AL . : "PURIFICATION OF A NEW , CALCIUM-INDEPENDENT, HIGH MOLECULAR WEIGHT PHOSPHOLIPASE A2/LYSOPHOSPHOLIPASE (PHOSPHOLIPASE B) FROM GUINEA PIG INTESTINAL BRUSH-BORDER MEMBRANE" cited in the application see the whole document ---	28
X	ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 306, no. 2, 1 November 1993, pages 534-540, XP000673197 DE CARVALHO, M.S., ET AL . : "THE 85-kDa, ARACHIDONIC ACID-SPECIFIC PHOSPHOLIPASE A2 IS EXPRESSED AS AN ACTIVATED PHOSPHOPROTEIN IN Sf9 CELLS" see the whole document ---	28
X	US 5 322 776 A (KNOPF JOHN L ET AL) 21 June 1994 see the whole document ---	13-16
P,X	US 5 466 595 A (JONES SIMON ET AL) 14 November 1995 see the whole document ---	1-5, 7-11, 13-19,28
P,X	US 5 554 511 A (JONES SIMON ET AL) 10 September 1996 see the whole document ---	1-5, 7-11, 13-19,28
E	US 5 589 170 A (JONES SIMON ET AL) 31 December 1996 see the whole document ---	1-5, 7-11, 13-19,28
T	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 13, 28 March 1997, pages 8567-8575, XP000673203 TANG, J., ET AL . : "A NOVEL CYTOSOLIC CALCIUM-INDEPENDENT PHOSPHOLIPASE A2 CONTAINS EIGHT ANKYRIN MOTIFS" see the whole document -----	1-28

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/17794

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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US 5322776 A	21-06-94	US 5354677 A	11-10-94
		US 5593878 A	14-01-97
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